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Regulation of Phospholipid Biosynthesis in the Yeast Saccharomyces Cerevisiae by the Ume6 Gene

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Regulation of Phospholipid Biosynthesis in the Yeast *Saccharomyces cerevisiae* by the *UME6* Gene

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LIST OF ABBREVIATIONS

A	adenine
aa	amino acid
ATP	adenosine triphosphate
bp	base pair
CAT	chloramphenicol acetyltransferase
C	cysteine
Ci	Curie
CTP	cytidine triphosphate
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
g	gram
GTP	guanosine triphosphate

HLH	helix-loop-helix
hr	hour (s)
IPTG	isopropyl-ß-D-thio-galactosidase
kb	kilobases
kDa	kilodalton
LB	Luria broth
L	liter
M	molar
mM	millimolar
min	minute (s)
ml	milliliter
µl	microliter
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PDME	phosphatidyldimethylethanolamine
PMME	phosphatidylmonomethylethanolamine
PI	phosphatidylinositol
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
RNA	ribonucleic acid

sec	second (s)
SDS	sodium dodecyl sulfate
TBE	tris-boric acid-EDTA
TE	tris-EDTA
tRNA	transfer RNA
TTP	thymidine triphosphate
U	units
UAS	upstream activation sequence
URS	upstream repression sequence
v	volts
vol	volumes

CHAPTER I

INTRODUCTION

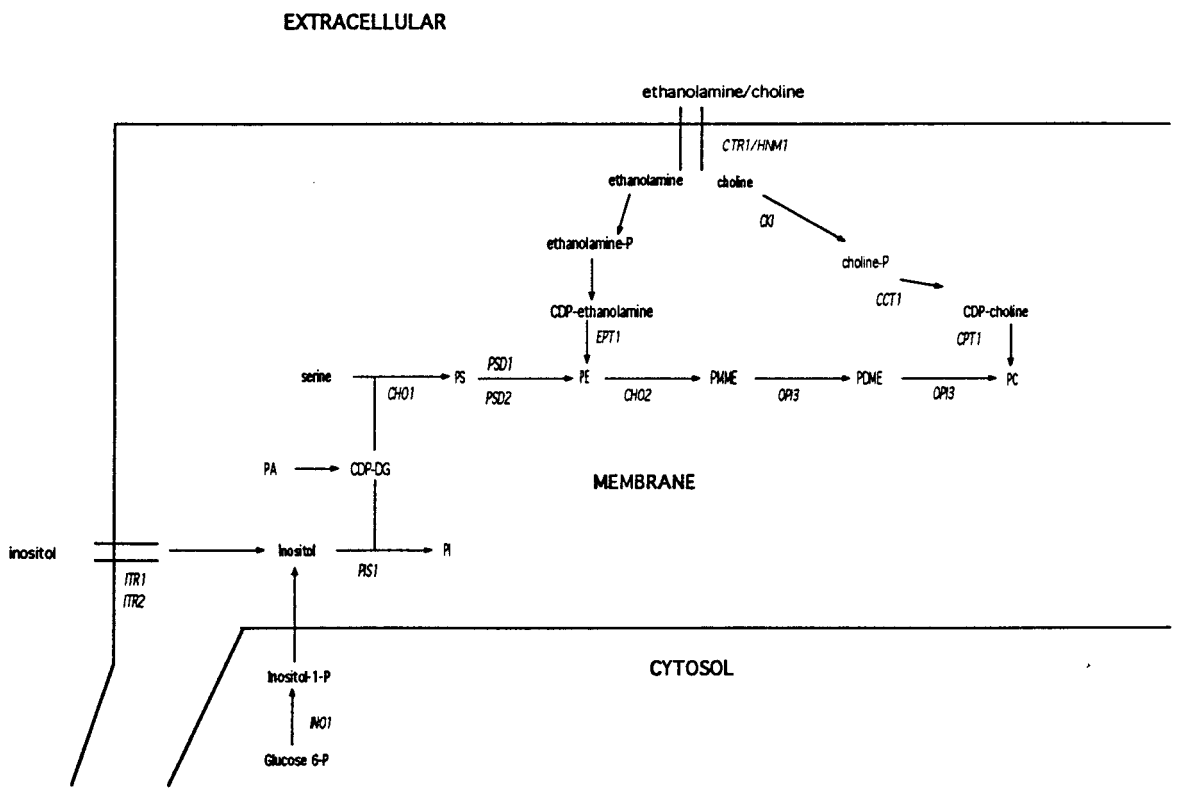
The yeast *UME6* gene product represses transcription of a diverse set of genes involved in meiosis (Strich *et al.*, 1994), heat shock response (Park and Craig, 1992), and nitrogen utilization (Park *et al.*, 1992). It also positively regulates expression of certain genes involved in meiosis (Bowdish and Mitchell, 1995). For all of these cases, the ability of *UME6* to regulate transcription requires the presence of a specific DNA sequence located within their respective promoters. Stimulated by the finding that the promoter of one of the phospholipid biosynthetic genes included the *UME6* cognate promoter sequence known as URS1, I examined the role of the *UME6* global regulatory gene in expression of membrane phospholipid biosynthetic genes. In the course of these studies, I discovered that the *UME6* gene product played both positive and negative roles in regulating phospholipid biosynthesis. The *UME6* gene product functioned directly as a transcriptional repressor through the URS1 element in the promoter of one phospholipid biosynthetic gene. Surprisingly, the *UME6* gene product also acted as a positive regulator of three other phospholipid biosynthetic genes, the promoters of which lack the URS1 element. I demonstrated that this positive regulation is indirect; it is the result of *UME6*-dependent regulation of a positive regulatory gene. As a result of its role as

both a positive and negative regulator of phospholipid biosynthetic gene expression, yeast strains which lack *UME6* exhibit a novel membrane phospholipid composition. To understand the role of *UME6* in regulating membrane biogenesis, I believe it is important to review the current understanding of both the pathway that permits the biosynthesis of membrane phospholipids and the regulatory cascade which controls this essential metabolic pathway.

The phospholipid biosynthetic pathway

The membranes of *Saccharomyces cerevisiae* are composed of phospholipids typical of eukaryotes and include phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylinositol (PI) (reviewed in: Carman and Henry, 1989, White *et al.*, 1991b). In *S. cerevisiae* as in other eukaryotes, phospholipids are synthesized in the membrane through a series of common reactions (Fig. 1). One branch of this pathway contains reactions that begin with phosphatidic acid (PA) and end with the synthesis of PC. The second reaction in this sequence converts cytidine diphosphate-diacylglycerol (CDP-DG) and free serine to PS and is catalyzed by phosphatidylserine synthase, a protein encoded by the *CHO1* gene (Bailis *et al.*, 1987). PS is subsequently converted to PE through a decarboxylation reaction catalyzed by two phosphatidylserine decarboxylases, the products of the *PSD1* and *PSD2* genes (Trotter *et al.*, 1993; Trotter and Voelker, 1995). In *S. cerevisiae*, methylation of PE is a major pathway for *de novo* synthesis of PC, and this process is catalyzed by the products of the *CHO2* and *OPI3* genes (Paltauf *et al.*, 1992; White *et al.*, 1991b).

Figure 1. The phospholipid biosynthetic pathway in *S. cerevisiae*.


EXTRACELLULAR
ethanolamine/choline
CTR1/HNM1
ethanolamine
choline
CKI
ethanolamine-P
choline-P
CCT1
CDP-ethanolamine
CDP-choline
EPT1
CPT1
serine
CHO1
PS
PSD1
PSD2
PE
CHO2
PMME
OPI3
PDME
OPI3
PC
PA
CDP-DG
MEMBRANE
inositol
ITR1
ITR2
Inositol
PIS1
PI
Inositol-1-P
INO1
Glucose 6-P
CYTOSOL

However, in a manner similar to other eukaryotes, *S. cerevisiae* can synthesize PC or PE from exogenously supplied choline or ethanolamine through the salvage pathway originally described by Kennedy and Weiss (Fig. 1) (Hjelmsted and Bell, 1987, 1988; Kennedy and Weiss, 1956). The first step in this pathway involves the rapid phosphorylation of choline or ethanolamine by choline kinase after transport into the cell by a choline transporter (encoded by the *CTR1* gene) (Li and Brendel, 1993). Currently, there is debate about the existence of separate enzymes for the phosphorylation of choline and ethanolamine, but experiments have shown that the enzyme encoded by the *CKI* gene (*i.e.*, choline kinase) is able to phosphorylate either substrate *in vivo* (Hosaka *et al.*, 1989). The second step in the salvage pathway is the conversion of phosphoethanolamine or phosphocholine to CDP-ethanolamine or CDP-choline which in the case of phosphocholine is catalyzed by the product of the *CCT1* gene (Tsukagoshi *et al.*, 1991). The final step converts CDP-ethanolamine and CDP-choline to PE and PC through a condensation reaction with diacylglycerol that is catalyzed by the products of the *EPT1* and *CPT1* genes, respectively (Hjelmsted and Bell, 1987; Hjelmsted and Bell, 1988; Hjelmsted and Bell, 1990).

The second branch of the phospholipid biosynthetic pathway results in the production of phosphatidylinositol (PI). PI can be synthesized *de novo* from glucose or from exogenous inositol (White *et al.*, 1991b). The two structural genes, *INO1* and *PIS1*, are required for the *de novo* synthesis of PI from glucose-6-phosphate. The *INO1* gene encodes the only cytosolic phospholipid biosynthetic enzyme, I1PS, which converts glucose-6-phosphate into inositol-1-phosphate

(Dean-Johnson and Henry, 1989; Klig and Henry, 1984). Inositol-1-phosphate is then rapidly dephosphorylated to inositol (Culbertson *et al.*, 1986; Culbertson *et al.*, 1986b). The membrane-bound *PIS1* gene product, phosphatidylinositol synthase, converts inositol and CDP-diacylglycerol into PI (Fischl and Carman, 1983; Fischl *et al.*, 1986). In order to produce PI from exogenous inositol, inositol must be transported into the cell, and this is accomplished by the product of the *ITR1* and *ITR2* genes which encode membrane-associated inositol transporter proteins (Lai and McGraw, 1994). Recent experiments have shown the *ITR1* gene to be transcribed at a substantially higher rate than the *ITR2* gene, and a mutation in *ITR1* almost completely eliminates inositol transport (Lai and McGraw, 1994; Nikawa *et al.*, 1991). Consistent with this observation, an *itr2* mutant strain has a modest defect in inositol transport (Lai and McGraw, 1994). Once transported into the cell, inositol is readily converted into PI by the product of the *PIS1* gene.

Control of enzymatic activity

The control of phospholipid biosynthesis in yeast occurs at several levels including: direct allosteric modulation of enzyme activity in response to soluble lipid precursors (Kelley *et al.*, 1988) and as a response to the phospholipid composition of the membrane (Fischl *et al.*, 1986; Hromy and Carman, 1986). The enzymatic activities of all the phospholipid biosynthetic genes in the pathway from PA to PC are inhibited in response to the precursors inositol and choline (Carson *et al.*, 1984; Homann *et al.*, 1985, 1987; Klig *et al.*, 1985; White *et al.*, 1991b; Yamashita *et al.*, 1982). In addition, I1P

synthase (encoded by the *INO1* gene) is also regulated in response to the presence of inositol and choline in the growth medium (Culbertson *et al.*, 1976; Donahue and Henry, 1981). The extent of the regulation among the enzymes varies, with I1P synthase being the most highly regulated at the level of over 30-fold (Culbertson *et al.*, 1976; Donahue and Henry, 1981). The activity of PI synthase is the only enzymatic activity in the pathway that has been shown to be unresponsive to inositol and choline (Fischl *et al.*, 1986).

Transcriptional control of phospholipid biosynthesis

Most of the phospholipid biosynthetic genes in yeast have been cloned, and this facilitated research concerning the regulation of phospholipid biosynthesis. Previous studies on the transcriptional regulation of phospholipid biosynthesis have focused primarily on *cis*- and *trans*-acting factors that either positively or negatively regulate this essential metabolic pathway. The *cis*-acting promoter elements include an upstream activation sequence and an upstream repression sequence. The function of these regulatory sequences is dependent on two activator proteins (the products of the *INO2* and *INO4* genes), and two repressor proteins (the products of the *OPI1* and *SIN3* genes).

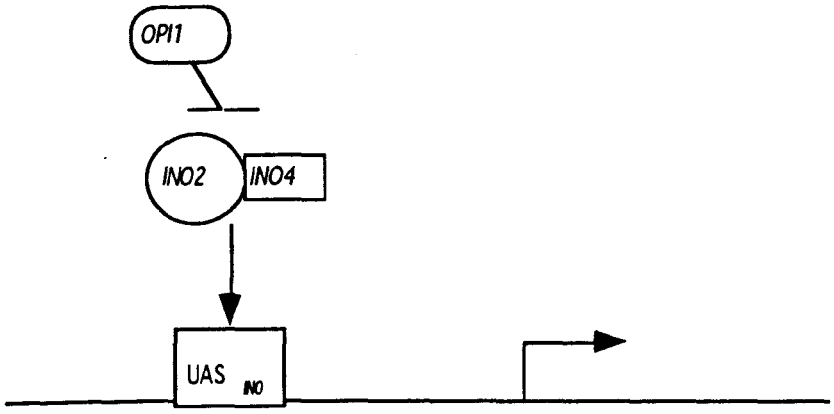
The UAS$_{INO}$ element

Regulation of the genes in the *de novo* pathway in response to inositol and choline has been shown to occur at the level of transcription of the *INO1* (Hirsch and Henry, 1986), *CHO1* (Bailis *et al.*, 1987), *CHO2* and *OPI3* (Gaynor *et al.*, 1991; Kodaki *et al.*, 1991),

structural genes. Recent work also has demonstrated that genes involved in the salvage pathway, including the inositol and choline/ethanolamine transporters *ITR1* (Lai and McGraw, 1994), *CTR1* (Li and Brendel, 1993) also are regulated at the level of transcription in response to inositol and choline. The coordinate regulation of these genes raised the possibility that their expression was controlled by a common *cis*-acting promoter element. In yeast, an upstream activation sequence (UAS) often serves as a binding site for transcriptional activator proteins which specify a response to a given growth signal. Through a combination of promoter deletion studies and DNA sequence analysis, a 10bp element called the UAS_{INO} was identified in the promoters of all the genes that are responsive to inositol and choline (Bachhawat *et al.*, 1995; Koipally *et al.*, 1995) (Fig. 2). However, while all yeast genes that are responsive to inositol and choline contain the UAS_{INO} element in their promoters, not all promoters which harbor a UAS_{INO} element are responsive to inositol and choline [for example, the *INO4* (Ashburner and Lopes, 1995) and *PIS1*(Anderson, 1996) genes].

The DNA sequence of the UAS_{INO} was determined through two experimental approaches. First, restriction fragments from the *INO1* (Lopes *et al.*, 1991) and *CHO1* (Bailis *et al.*, 1992) promoters were fused to a *CYC1-lacZ* reporter gene, and tested for their ability to activate transcription of this reporter gene. These experiments revealed that every fragment capable of conferring inositol-specific regulation contained a similar promoter element with a derived consensus sequence of 5' CATGTGAAAT 3'. The possibility that this sequence was the UAS_{INO} element was tested formally by inserting a

Figure 2. Model for the transcriptional regulation of phospholipid biosynthetic genes. Opi1p interacts with the Ino2p:Ino4p heterodimer to inhibit its ability to activate transcription through the UAS_{INO} element (refer to text for details).



Phospholipid Biosynthetic Genes

INO1
CHO1
CHO2
OPI3
CKI
CPT1
CTR1
ITR1

synthetic oligonucleotide containing this sequence upstream of a *CYC1-lacZ* reporter gene. This experiment confirmed that this 10bp sequence is sufficient for the inositol/choline response (Bachhawat *et al.*, 1995; Koipally *et al.*, 1995).

As is the case with other UAS elements, the specific DNA sequence of the element is vital for its function. Experiments have demonstrated that substitutions at the first six positions of the UAS_{INO} element severely affect or eliminate its function (Bachhawat *et al.*, 1995). Recent experiments have also demonstrated that the UAS_{INO} element serves as a binding site for a heterodimer of the Ino2p and Ino4p proteins (Ambroziak and Henry, 1994; Nikoloff and Henry, 1994), which belong to the family of helix-loop-helix (HLH) proteins (Hoshizaki *et al.*, 1990; Nikoloff and Henry, 1991; Nikoloff *et al.*, 1992). The necessity for DNA sequence identity within the first six nucleotides of the UAS_{INO} element is not surprising since these bases correspond to the general binding site for HLH proteins (5' CANNTG 3') (Blackwell and Weintraub, 1990).

The *INO2* and *INO4* positive regulatory genes

Derepression of genes in the phospholipid biosynthetic pathway in response to inositol and choline deprivation absolutely requires the products of the *INO2* and *INO4* genes (Bailis *et al.*, 1987; Hirsch and Henry, 1986; Klig *et al.*, 1988; Nikoloff *et al.*, 1992). The *INO2* and *INO4* genes were originally isolated in a mutagenic screen for inositol auxotrophs and comprised two of the ten complementation groups (Culbertson and Henry, 1975; Donahue and Henry, 1981). The requirement of inositol for growth of the *ino2* and

ino4 strains is the same as in an *ino1* strain since the *ino2* and *ino4* strains fail to express the *INO1* gene (Donahue and Henry, 1981b). Subsequent studies revealed that strains harboring mutant alleles of *ino2* or *ino4* suffered from a pleiotropic defect in phospholipid biosynthesis. The inositol requirement of *ino2* and *ino4* mutant strains provided a convenient genetic screen that was used to clone the genes. Both the *INO2* and *INO4* genes were cloned by their ability to complement the inositol growth requirement and to restore expression of the *INO1* gene (Nikoloff *et al.*, 1992; Klig *et al.*, 1988). Subsequent analysis identified a 453bp open reading frame in the *INO4* clone and a much larger 912bp open reading frame in the *INO2* clone (Hoshizaki *et al.*, 1991; Nikoloff *et al.*, 1992).

Analysis of the proteins predicted by the sequence of the *INO2* and *INO4* genes revealed homology to the family of basic helix-loop-helix proteins (Nikoloff *et al.*, 1991; Hoshizaki *et al.*, 1991), which are known to form dimers in order to bind DNA and activate transcription (Murre *et al.*, 1989). The prediction that Ino2p and Ino4p form a heterodimer to bind DNA was reinforced by the results of mobility shift experiments. These experiments revealed DNA:protein complexes on the *INO1* promoter that were dependent on wild type alleles of both the *INO2* and *INO4* genes (Lopes *et al.*, 1991). Recently, this interaction has been studied more intensively. Experiments using an *INO2*-specific antibody revealed the presence of Ino2p in the previously described protein:DNA complexes (Nikoloff and Henry, 1994). Further studies using Ino2p and Ino4p produced in *E. coli* have showed that these two proteins form a heterodimer independent of DNA and still form the same protein:DNA complexes

originally identified in the mobility shift experiments (Ambroziak and Henry, 1994; Schwank *et al.*, 1995). Furthermore, the requirement for the HLH domain in formation of the Ino2p:Ino4p heterodimer has been demonstrated using the two-hybrid system and through Far-western analysis (Schwank *et al.*, 1995). The DNA binding specificity of the Ino2p:Ino4p heterodimer was further resolved by demonstrating that the heterodimer, synthesized *in vitro*, specifically bound to an artificial consensus UAS_{INO} element, but not to an artificial UAS_{INO} element which varied from the consensus (Ambroziak and Henry, 1994). The transcriptional activation function of the Ino2p:Ino4p heterodimer is dependent on two regions of the amino-terminus of Ino2p, while Ino4p does not have the ability to activate transcription (Schwank *et al.*, 1995). Therefore, Ino2p activates transcription by binding to the UAS_{INO} element, but only after dimerization with Ino4p (Fig. 2).

Autoregulation of *INO2* expression

The possibility existed that regulation of phospholipid biosynthesis occurred through the regulation of the *INO2* and/or *INO4* activator gene expression. This possibility was supported by two lines of evidence: the promoters of the *INO2* and *INO4* genes contain copies of the UAS_{INO} element; and reduced levels of the Ino2p:Ino4p:UAS_{INO} complex were formed when extracts were used from cells grown under repressing conditions (Lopes and Henry, 1991).

The transcriptional regulation of the *INO2* and *INO4* genes was analyzed by fusing their promoters to the *cat* reporter gene

(Ashburner and Lopes, 1995). The results from these experiments revealed that expression of *INO2-cat* closely resembles the expression pattern of its target genes. Expression of *INO2-cat* is induced approximately 10-fold in the absence of inositol and choline, absolutely requires the products of both the *INO2* and *INO4* genes, and *INO2* is constitutively overexpressed in an *opi1* mutant strain (Ashburner and Lopes, 1995). In contrast, expression of the *INO4-cat* is constitutive and requires the product of the *INO4* gene, not *INO2* (Ashburner and Lopes, 1995). In addition, *INO4-cat* is overexpressed in comparison to *INO2-cat*, suggesting that Ino2p may be limiting in respect to Ino4p (Ashburner and Lopes, 1995). This prediction is supported by experiments demonstrating that the presence of *INO2* on a multicopy plasmid results in higher than normal expression of *INO1* under repressing conditions (Hosaka *et al.*, 1994) and leads to increased formation of an Ino2p:Ino4p:UAS$_{INO}$ complex in mobility shift assays (Nikoloff and Henry, 1994). Recent experiments have also demonstrated that the levels of the native *INO2* and *INO4* transcripts are regulated in an identical fashion as the *INO2-cat* and *INO4-cat* constructs (J. Lopes, unpublished results).

Taken together, the previously cited experiments raised the possibility that regulation of phospholipid biosynthesis occurred through regulation of the *INO2* activator gene. This possibility was examined by using a yeast strain that contained the *INO2* gene under the control of the *GAL1* promoter (Ashburner and Lopes, 1995b). In this strain, *INO2* is induced at high levels in the presence of galactose, is repressed in the presence of glucose, but is unresponsive to inositol and choline (Ashburner and Lopes, 1995b). The results of

these studies show that transcription of *INO1* and *CHO1* are still responsive to inositol and choline even though transcription of the *INO2* gene is not responsive to inositol (Ashburner and Lopes, 1995b). However, the level of *INO1* and *CHO1* expression is dependent on the level of *INO2* transcription, indicating that the regulation of *INO2* serves to determine the level of target gene expression (Ashburner and Lopes, 1995b).

The *OPI1* negative regulatory gene

The product of the *OPI1* gene is absolutely required for repression of the phospholipid biosynthetic genes in response to exogenously supplied inositol and choline. The *OPI1* gene was originally identified using a bioassay for mutants that excreted inositol into the growth medium (Greenberg *et al.*, 1982). The inositol excretion phenotype has not been fully resolved; however, it is most likely due to overproduction of the *INO1* gene (Hirsch and Henry, 1986). Subsequent research revealed that in addition to the *INO1* gene, transcripts of the *CHO1* (Bailis *et al.*, 1991), *CHO2* and *OPI3* genes are all constitutively overexpressed in an *opi1* mutant strain (Jackson and Lopes, in press). These results indicate that the *OPI1* gene is involved in transcriptional regulation of phospholipid biosynthesis.

Cloning and analysis of the *OPI1* gene revealed that the putative Opi1p contains motifs common to regulatory proteins. Originally, *OPI1* was genetically mapped and found to be closely linked to the *SPO11* gene (White *et al.*, 1991). Genomic clones containing the region around *SPO11* were screened for their ability to

complement a defect in an *opi1* strain. Specifically, the genomic clones were tested for their ability to properly regulate an *INO1-lacZ* reporter and to eliminate the inositol excretion phenotype (White *et al.*, 1991). An examination of the Opi1p predicted protein sequence revealed two features common to proteins involved in transcriptional regulation: a leucine zipper, and a glutamine rich region (White *et al.*, 1991). The leucine zipper domain was originally identified as a dimerization domain in the mammalian C/EBP protein (Landschulz *et al.*, 1988), and dimerization of C/EBP is absolutely required for its binding to DNA (Landschulz *et al.*, 1989). However, it is not known currently if Opi1p acts as a dimer, or if it even binds DNA. The glutamine-rich regions of Opi1p are interesting because they may be involved in protein:protein interactions (Gerber *et al.*, 1984).

Despite the phenotype of an *opi1* mutant strain and the information provided by the sequence of the *OPI1* gene, it is still not clear how *OPI1* functions to regulate phospholipid biosynthetic gene expression. However, recent experiments demonstrate that an *opi1* mutant strain constitutively overexpresses a *CYC1-lacZ* heterologous reporter gene under the control of the UAS_{INO} element, indicating that *OPI1* functions to regulate phospholipid biosynthesis through the UAS_{INO} element (Bachhawat *et al.*, 1995; Koipally *et al.*, 1995). Unfortunately these results do not provide evidence for a direct interaction between Opi1p and the UAS_{INO}. One other possibility was that Opi1p regulated *INO1* transcription through the UAS_{INO} element indirectly by regulation of the *INO2* activator gene (Ashburner and Lopes, 1995). This possibility is ruled out by experiments which demonstrate that expression of *INO1* and *CHO1* is not responsive to

inositol in an *opi1* mutant strain when *INO2* is expressed constitutively using the *GAL1* promoter (Ashburner and Lopes, 1995b). These experiments suggest that *OPI1* must regulate *INO1* and *CHO1* directly. The current model for *OPI1* function predicts that the Opi1p interacts transiently with either Ino2p or the Ino2p:Ino4p complex as a whole (Fig. 2). In support of this model, the presence of polyglutamine tracts in Opi1p suggest potential protein:protein interaction domains. In addition, all of the *opi1* mutant alleles that have been sequenced so far predict an Opi1p truncated within these polyglutamine tracts (White *et al.*, 1991). Clearly, further research is necessary to clarify the mechanism of *OPI1* function.

URS1-dependent regulation and the *SIN3* negative regulatory gene

Many yeast genes contain negative regulatory sequences in their promoter regions, and these sequences are referred to as upstream repression sequences (URS). One such URS element is the URS1 (5' AGCCGCCGA 3'), a copy of which is found in the promoter of the *INO1* gene (Lopes *et al.*, 1993). The URS1^{INO1} element in the *INO1* promoter is a perfect match to the consensus URS1 sequence and a large deletion which removed this element resulted in a substantial increase in the expression of an *INO1-lacZ* reporter gene (Lopes *et al.*, 1993).

Since the product of the *SIN3* gene is often involved in repression mediated by the URS1 element, it is not surprising that *SIN3* was isolated in a mutant screen for genes involved in repression of *INO1* transcription. The defect in *INO1* expression was

identified by a genetic screen for mutants that expressed an *INO1-lacZ* reporter construct under repressing conditions. Three allelic mutants were isolated and were designated constitutive phospholipid expression (*cpe1*) mutants (Hudak *et al.*, 1994). Later, it was shown that *cpe1* was allelic to other mutants (*rpd1*, *ume4*, *gam2*, *sdi1*) and that all these represent the same genetic locus, the *SIN3* locus (Hudak *et al.*, 1994). Consequently, all of these mutations are now designated as *sin3* mutations since this is the original name for mutations at this locus.

The role of *SIN3* in repression of the phospholipid biosynthetic genes has been examined using two strategies. First, the effect of a *sin3* mutation was examined by quantitating expression of the phospholipid biosynthetic genes using Northern blot hybridizations. Surprisingly, mutations at *sin3* pleiotropically affected expression of the phospholipid biosynthetic genes (Hudak *et al.*, 1994). This was surprising because of the genes tested (*INO1*, *CHO1*, *CHO2*, *OPI3*), only the *INO1* promoter was known to contain a URS1 element (Hudak *et al.*, 1994). The second strategy was to determine the effect of a *sin3* null mutation on expression of various fusions of the *INO1* promoter to the *CYC1-lacZ* reporter gene (Slekar and Henry, 1995). Three basic constructs were assayed: some contained the UAS_{INO} and the $URS1^{INO1}$ elements; some contained just the UAS_{INO} element; and some contained an artificial consensus UAS_{INO} element. The results demonstrated that *SIN3* affects expression from both the sole $URS1^{INO1}$ element as well as from the UAS_{INO} elements. This explains the pleiotropic effect on repression of genes which lack a $URS1^{INO1}$ element but contain the UAS_{INO} element (Slekar and Henry, 1995).

CHAPTER II

REVIEW OF THE RELATED LITERATURE

The expression of some yeast genes has been shown to be controlled by repressors that specifically interact with promoter elements called upstream repressor sequences (URS) (Brent, 1985; Levine and Manley, 1991). For example, repression of the *GAL1* gene in response to glucose is established by URS elements found in the promoters of the *GAL1* and *GAL4* genes (Flick and Johnston, 1990; Griggs and Johnston, 1991). Several other systems have been shown to be under the control of a sequence generally called the URS1 element (5' AGCCGCCGA 3') , namely the *HO* (Sternberg *et al.*, 1987), *CAR1* (Luche *et al.*, 1990), and *SPO13* (Buckingham *et al.*, 1990) genes. However, the function of the URS1 element is most likely not limited to these three genes since a number of other yeast genes, including the *INO1* gene (Lopes *et al.*, 1993), share a promoter sequence similar to the URS1 element (Luche *et al.*, 1990). The *SIN3*-mediated regulation of the phospholipid biosynthetic genes and the *SIN3*/URS1-mediated repression of the *INO1* gene prompted this study of *UME6* regulation of phospholipid biosynthesis because the *UME6* gene is often required for URS1-mediated gene regulation. Since the role of *SIN3* in the regulation of phospholipid biosynthesis has already been examined and is not the focus of this study, I will only briefly review the literature concerning *SIN3* function. In the

rest of this chapter, I will more extensively review the role of *UME6* in the regulation of nitrogen catabolism and meiosis. To facilitate review of *SIN3*/*UME6*/URS1 regulatory systems, I have provided a summary figure (Fig. 3) and paragraph at the end of this chapter.

The *SIN3* global negative regulator

The ability of the URS1 element to repress gene transcription in some systems is dependent on the product of the *SIN3* gene. For example, repression of *HO* expression has been shown to be dependent on the *SIN3* gene although there is no evidence of *SIN3* binding directly to the *HO* promoter (Wang *et al.*, 1990; Wang and Stillman, 1993). The *SIN3*-mediated repression of *HO* has been shown to require a protein, Sdp1, which recognizes the URS1^{HO} element (Wang and Stillman, 1990). The *SIN3* gene is also required for repression of the *SPO13* gene (Strich *et al.*, 1989) and the *TRK2* gene (Vidal *et al.*, 1991). The promoters of the *SPO13* and *TRK2* genes both contain URS1 elements, although, recent experiments indicate that *SIN3* does not work through the URS1 element in the *TRK2* promoter (Vidal *et al.*, 1995). Since *SIN3* often works through the URS1 element which is found in a diverse set of yeast promoters, it is not surprising that it was identified through a number of different genetic screens. The *SIN3* gene was identified by genetic screens for defects in: *HO* expression (Sternberg *et al.*, 1987); early meiotic gene expression (*UME4*) (Strich *et al.*, 1989); potassium uptake (*RPD1*/*SDI1*) (Vidal *et al.*, 1991); extracellular glucoamylase production (*GAM2*) (Yoshimoto *et al.*, 1992); and *INO1* expression (*CPE1*) (Hudak *et al.*, 1994) (see above).

UME6 regulation of nitrogen catabolism

When haploid yeast cells are confronted with a limited supply of nitrogen in their environment, several biosynthetic pathways are repressed, such as arginine biosynthesis, while a number of catabolic pathways, such as those involved in degradation of arginine, are induced (rev. in: Magasanik, 1993). Catabolism of arginine requires the products of the *CAR1* and *CAR2* genes, which encode arginase and ornithine transaminase, respectively (rev. in: Magasanik, 1993). The *CAR1* gene is repressed by efficiently utilized nitrogen sources (such as, ammonia, glutamine, or asparagine) while in the absence of nitrogen, both *CAR1* and *CAR2* are derepressed (Dubois *et al.*, 1974). Expression of the *CAR1* gene has been extensively studied and provides an excellent example of *UME6* function. The *CAR1* gene is only expressed when arginine is available within the cell and this expression is mediated by three major UAS elements (Kovari *et al.*, 1990) and one upstream repression sequence (URS1) (Sumrada and Cooper, 1987; Luche *et al.*, 1990). Two UAS elements, UAS_{C1} and UAS_{C2}, mediate inducer-independent expression and are repressed by the stronger negative regulatory site, URS1 (Kovari *et al.*, 1990; Luche *et al.*, 1990). Therefore, in wild type cells, the balance between activation and repression of *CAR1* is tipped in the favor of reduced *CAR1* expression. However, when arginine is present, the third UAS, UAS_I, which is inducer dependent, joins the other two UASs, and the combination of the three UAS elements is enough to overcome the URS1-mediated repression, and *CAR1* transcription is increased (Viljoen *et al.*, 1992).

The *CAR1* URS1 element was originally identified by a single point mutation (CAR1-*O*$^-$) which rendered *CAR1* expression constitutive (Sumrada and Cooper, 1985). Saturation mutagenesis demonstrated that the URS1 element is a 9bp sequence, 5' AGCCGCCGA 3' which bound a specific protein(s) (Luche *et al.*, 1990). Studies in many laboratories identified the URS1 element in the promoters of several unrelated genes, including those involved in: sporulation (Malavasik and Elder, 1990; Engebrecht and Roeder, 1990); mating type specification (Wang and Stillman, 1990); heat shock response (Park and Craig, 1990); oxidative metabolism (Spevak *et al.*, 1983) and inositol metabolism (Lopes *et al.*, 1993).

The presence of the URS1 element in the promoters of several unrelated genes raised the possibility that a common *trans*-acting factor(s) may be associated with it. Earlier studies had identified a locus (*car80* [*cargRI*]) unlinked to *CAR1* which generated a phenotype similar to the *CAR1-O*$^-$ mutant (Wiame, 1971), indicating that *CAR80* may be a factor required for URS1 function. Experiments carried out in the laboratory of Dr. Terrance Cooper suggested that *CAR80* is allelic to *UME6*. Using a *CYC1-lacZ* reporter system that contained the URS1^{CAR1}, Cooper demonstrated that *ume6* and *car80* mutant strains had a similar defect in repression of the reporter construct (Park *et al.*, 1992). In addition to the loss of URS1^{CAR1} function in a *ume6* mutant strain, a *ume6* mutant strain also has a decreased frequency of sporulation. In a more definitive test of allelism, a *ume6*Δ strain was crossed to a wild type strain (*CAR80*) and a *car80* mutant strain, and the resulting diploids were sporulated. The wild type *CAR80* allele fully complemented the *ume6*Δ mutation, while the *car80*

mutant allele failed to complement the *ume6*Δ sporulation defect (Park *et al.*, 1992). In a similar fashion, only the wild type *CAR80* allele effectively complemented the *ume6*Δ mutation in the URS1-*CYC1-lacZ* reporter assay (Park *et al.*, 1992). Taken together, these experimental results indicate that *CAR80* is allelic to *UME6*. More recently, sequence analysis of the cloned *UME6* and *CAR80* genes has indicated that they are identical (Strich *et al.*, 1994).

As discussed earlier, expression of the catabolic genes *CAR1* and *CAR2* is repressed by nitrogen. Currently, three lines of evidence exist which indicate the importance of *UME6* for this repression. First, mutants containing a point mutation or disruption of *UME6* have derepressed levels of arginase (*CAR1*) and ornithine transaminase (*CAR2*) activity when the strains are grown in medium containing ammonia as a nitrogen source (Strich *et al.*, 1994). Secondly, in a *ume6*Δ strain, the level of derepression of the *CAR1* and *CAR2* genes is comparable with wild type levels under nitrogen starvation conditions (Strich *et al.*, 1994). Lastly, when a *ume6*Δ mutant strain is starved for nitrogen, only a slight increase in *CAR1* and *CAR2* gene expression occurs (Strich *et al.*, 1994), demonstrating that repression of the *CAR1* and *CAR2* genes is the primary means of controlling nitrogen catabolism.

The requirement of the *UME6* gene product for URS1-mediated repression raised the possibility that *UME6* might encode the URS1 binding protein. Using the URS1 element in an affinity column, Luche *et al.*, purified a heteromeric protein complex which bound specifically to the URS1 element (1992). The two polypeptides had molecular masses of 37.5 and 73.5 kDa, were designated Buf1p and

Buf2p respectively, and experiments demonstrated that the smaller species was not a degradation product of the larger species (Luche *et al.*, 1992). Antibodies were raised to the purified Buf proteins and used to screen a λgt11 expression library in order to clone their respective genes. Once cloned, an attempt was made to disrupt the *BUF1* and *BUF2* genes, and determine their resulting phenotypes. This proved unsuccessful in both cases, indicating that the *BUF1* and *BUF2* genes are essential (Luche *et al.*, 1993). A search of protein data bases revealed that the deduced Buf1p and Buf2p protein sequences were identical to the heteromeric RF-A (RP-A) protein which is a component of the DNA replication apparatus (Luche *et al.*, 1993). Replication factor or protein A is a trimeric protein consisting of 69, 36, and 13 kDa subunits, and its principle known function is as a single-stranded DNA binding protein (Heyer *et al.*, 1990; Brill and Stillman, 1991). Subsequent experiments revealed that the purified Buf protein complex had a higher affinity to a double stranded oligonucleotide containing the URS1 element than to a single stranded oligonucleotide (Luche *et al.*, 1993). When the purified Bufp protein complex was subjected to SDS-PAGE under conditions that would allow the identification of a 14 kDa protein, a third component of the Buf complex was identified that comigrated with a 14 kDa standard and found to be identical to the smallest subunit of RF-A (RP-A) (Luche *et al.*, 1993). While the role of the RF-A (RP-A) complex in transcriptional control has not been determined, it may represent a general DNA-binding factor, that in combination with *UME6*, combines to regulate *CAR1* and *CAR2* expression.

UME6 control of early meiotic gene expression

Cells of *S. cerevisiae* divide mitotically when nutrients are plentiful; however, starvation causes cell growth and mitotic division to cease. An a/α diploid cell will undergo a program of sporulation that leads through meiosis and to spore formation, while a or α haploid cells become arrested at the G_1 phase of the mitotic cell cycle (rev. in: Honigberg *et al.*, 1993). Two nutritional conditions are required for sporulation. One condition is limitation for an essential nutrient such as nitrogen, and another is absence of a fermentable carbon source, such as glucose.

Genes involved in meiosis have been divided into three classes, based on their time of expression. Early meiotic genes are expressed at the beginning of meiotic prophase which includes a round of DNA synthesis and events associated with recombination (*i.e.*, chromosome condensation, transient double-stranded chromosome breaks, and gene conversion) (rev. in: Honigberg, *et al.*, 1993). middle genes are expressed later in prophase where meiosis I (reductional) and meiosis II (equational) divisions occur (rev. in: Honigberg, *et al.*, 1993). Late genes are expressed around the time of meiotic divisions and spore packaging (rev. in: Honigberg, *et al.*, 1993).

The initiation of meiotic development is controlled by signal transduction pathways that monitor both glucose and nitrogen levels and interact with an independent pathway responding to cell type. Together, these pathways regulate transcription of a major inducer of meiosis, *IME1*, a meiosis-specific transcriptional activator (Smith *et al.*, 1993). In vegetatively growing diploid cells, the product of the

RME1 gene represses *IME1* transcription, and this inhibits meiosis; however, under conditions of starvation, the a1-α2 negative regulator inhibits transcription of *RME1*, allowing *IME1* transcription and consequently meiosis, to occur (Mitchell and Herskowitz, 1986; Kassir *et al.*, 1988; Covitz *et al.*, 1989). After induction of *IME1* transcription, a complex regulatory pattern controls both the onset and duration of expression of most meiotic genes.

Previous results demonstrating that the *SPO13* gene was induced early in meiosis (Wang *et al.*, 1987), allowed for a convenient screen to isolate mutants involved in the regulation of early meiotic genes. Originally, a haploid yeast strain containing a *SPO13-lacZ* fusion was mutagenized, and mutants were isolated that expressed the *SPO13-lacZ* fusion on media containing both glucose and nitrogen (Strich *et al.*, 1989). Five different mutants were obtained and designated *ume* mutants (1-5) (unscheduled meiotic gene expression). Subsequent analyses revealed not only an increase in *SPO13* mRNA levels during vegetative growth, but also an increase in levels of other early meiotic genes, such as *SPO11* and *SPO16*, that was independent of *IME1* levels (Strich *et al.*, 1989). Further experiments have revealed that *UME1, 2, 3,* and *5* are involved in glucose repression and rapid mRNA turnover of meiotic genes (Surosky and Esposito, 1992; Surosky *et al.*, 1994), while the *UME4* gene has been shown to be identical to *SIN3* (*RPD1, CPE1, SDI1, GAM2*), a gene involved in transcriptional repression (Vidal *et al.*, 1991).

To identify genes required for the degradation of early meiotic mRNAs, a second search was initiated for mutants that would

continue to express a *SPO13-lacZ* fusion when meiotic cells were returned to rich growth media. This strategy resulted in the isolation of another *ume* mutant, *UME6*, that expressed constitutively high levels of β-galactosidase activity even without prior meiotic induction (Strich *et al.*, 1994). This result suggested that *UME6* was involved in the vegetative repression of *SPO13*, not in mRNA turnover after meiotic induction. Further analysis revealed that a *ume6* mutant strain displays a 70-fold induction of early meiosis-specific genes during vegetative growth, while the previously identified *ume* mutants exhibit a maximum 10-fold derepression (Strich *et al.*, 1994). The *ume6* gene was cloned by complementation of the *ume6* mutant phenotype allowing constitutive expression of a *SPO13-lacZ* fusion, and subcloning of the putative *UME6* gene indicated the same region could complement a *car80* mutation (Strich *et al.*, 1994). Subsequent physical and genetic mapping placed *UME6* on the right arm of chromosome IV, between *pet14* and *hom2*, and linked to *RAD9* (Strich *et al.*, 1994).

Independent fragments which complemented *ume6* and *car80* mutations were sequenced, shown to be identical, and contained a single open reading frame encoding a 91-KD protein of 836 amino acids (Strich *et al.*, 1994). Computer-assisted searches of protein databases revealed homology in Ume6p to the C6 zinc-cluster DNA-binding domain found in a number of regulatory proteins including Gal4p, Hap1p, and Arg81p (Strich *et al.*, 1994). To determine if the C6 region of Ume6p is important for its function, amino acids known to be important for Gal4p function were altered by site-directed mutagenesis. Crystallographic analysis of Gal4p has revealed that

Lys-18 forms multiple sequence specific bonds to the Gal4p binding site and Cys-14 participates in zinc-binding (Marmorstien, 1992). In Ume6p, Cys-774 corresponds to Gal4p Cys-14, and Lys-778 corresponds to Gal4p Lys-18 (Strich *et al.*, 1994). Experiments using a *SPO13-lacZ* fusion gene demonstrated that a mutation of either Cys-774 or Lys-778 in Ume6p results in unregulated expression of the fusion gene (at levels similar to a *ume6Δ* mutant), indicating the importance of the C6 motif in Ume6p function (Strich *et al.*, 1994). One easy mechanism for regulating Ume6p function would be to regulate the expression of *UME6*, and this possibility was examined through Northern blot analysis. Under either conditions of nitrogen starvation or meiosis, the 2.7kb *UME6* mRNA was shown to be constitutively expressed, demonstrating that Ume6p activity is not under transcriptional control (Strich *et al.*, 1994).

Since *UME6* is required for the proper regulation of many early meiotic genes, it seemed plausible that the promoters of these genes may contain a common *cis*-acting sequence(s) required for *UME6* regulation. Detailed studies of the 5' regions of the early meiotic genes *SPO13*, *HOP1*, and *IME2* allow four general conclusions to be drawn (Bowdish and Mitchell, 1993; Buckingham *et al.*, 1990; Vershon *et al.*, 1992). First, these genes contain the URS1 negative regulatory element in their promoters. Second, the URS1 element represses early meiotic genes in non-meiotic cells but stimulates these promoters during meiosis. Third, stimulation dependent on the URS1 element often requires the presence of another nearby promoter element. Finally, many promoters of early meiotic genes

have regulatory elements located in close proximity to their TATA box and RNA start site(s).

Analysis of the *SPO13* promoter first implicated the URS1 site in meiosis-specific expression. Through the use of a *SPO13-lacZ* fusion containing 185bp of the *SPO13* promoter, Buckingham *et al.*, demonstrated that this fusion was regulated in a meiosis specific fashion (1990). Deletion analysis of the *SPO13* promoter revealed that a point mutation in the URS1 element or removal of the URS1 element yielded two consequences: a 6-fold decrease in expression during meiotic induction, and a slight elevation in expression in non-meiotic cells, suggesting that the URS1 site in the *SPO13* promoter may have a positive role during meiosis and act as a negative site in non-meiotic cells (Buckingham *et al.*, 1990). Similar studies of the *HOP1* and *IME2* promoters have yielded the same conclusions regarding the function of the URS1 element (Vershon *et al.*, 1992; Bowdish and Mitchell, 1993).

Epistasis analysis of strains carrying a *ume6* mutation and a mutation in the URS1 element indicated that the double mutants have a similar level of *SPO13* derepression during vegetative growth as strains carrying a single mutation, indicating that Ume6p works through the URS1 element (Strich *et al.*, 1994). To further investigate this interaction, electrophoretic mobility shift assays (EMSA) were used to compare the protein:DNA complexes formed from a URS1^{SPO13} containing probe and cell extracts from wild type (*UME6*) and *ume6*Δ mutant strains. These experiments revealed that extracts from the *ume6*Δ mutant strain failed to form two of the DNA:protein complexes found using the wild type cell extracts,

suggesting that *UME6* regulates *SPO13* expression through protein interactions at the URS1^{SPO13} element (Strich *et al.*, 1994). To determine if the Ume6p:URS1^{SPO13} interaction is direct or indirect, the carboxy-terminal third of Ume6p including the C6 DNA-binding domain was fused to the amino terminal portion of the *E. coli* maltose binding protein (MBP) to allow for production in *E. coli* and easy purification. The MBP-Ume6p fusion protein was used with the URS1^{SPO13} DNA probe in EMSAs. The MBP-Ume6p fusion protein produced a significant shift in DNA probe migration, whereas MBP alone did not (Strich *et al.*, 1994). In addition, experiments indicate that the interaction does not require the presence of additional yeast proteins or yeast-specific protein modifications (Strich *et al.*, 1994). This result seems in contradiction with that demonstrated for the regulation of the arginase gene *CAR1*, where the RF-A (RP-A) complex appears to bind the URS1 element directly while Ume6p does not (Luche *et al.*, 1993). One explanation for the apparent contradiction is that the RF-A (RP-A) complex may represent a general DNA-binding factor that works in conjunction with Ume6p to establish the expression pattern of *CAR1* and not function in the regulation of early meiotic gene expression.

The recent cloning of the *UME6* gene has facilitated biochemical studies of the Ume6p protein. Through the use of ^{113}Cd NMR and comparison to proteins known to contain the C6 DNA binding motif, such as Gal4p, experiments have demonstrated that the six cysteine residues in the C6 domain of Ume6p coordinate the zinc atoms required to form a binuclear metal cluster (Anderson *et al.*, 1995). Surprisingly, the binuclear zinc cluster in Ume6p accounts for most of

CD spectrum observed for Ume6p, and it is likely that little other secondary structure exists in the Ume6p, in marked contrast to Gal4p which contains significant organized secondary structure outside the binuclear zinc cluster (Anderson *et al.*, 1995). Binding of Ume6p to the URS1 element was also mapped using DNase I protection assays. These experiments revealed that both the full length Ume6p as well as the C-terminal 111 amino acids (contains the C6 domain) interact with the DNA roughly in the center of the 5' CCGCCG 3' sequence (Anderson *et al.*, 1995). Proteins containing the binuclear zinc cluster domain often interact with CCG or CGG triplets, although for Ume6p the binding site is a direct repeat without a spacer, a previously undiscovered arrangement (Anderson *et al.*, 1995). In the case of other binuclear zinc cluster containing proteins, such as Gal4p, the spacer is required to properly coordinate the binding of a homodimer to the DNA (Marmorstein *et al.*, 1992); however, experimental evidence from DNase I protection assays and EMSAs suggest that Ume6p interacts with DNA as a monomer (Anderson *et al.*, 1995).

When cells enter meiosis, expression of *IME1* increases, and *IME1* is required for expression of nearly all meiotic genes (rev. in Mitchell, 1994). As discussed above, mutations in the URS1 element or deletions of the URS1 element in the promoters of early meiotic genes reduced their expression during meiosis (Buckingham *et al.*, 1990; Vershon *et al.*, 1992; Bowdish and Mitchell, 1993). Combined with a recent observation that *UME6* is required for *IME1*-dependent activation of *IME2* (Bowdish and Mitchell, 1993), this suggests that the URS1/*UME6* system is required for *IME1* to activate early meiotic

genes. In a study of *IME1*-dependent activation, a mutant, *rim16-12*, was obtained which impaired the ability of *IME1* to activate transcription and activate sporulation (Bowdish and Mitchell, 1993; Mitchell and Bowdish, 1992). Further study of this mutant revealed that it actually was a *ume6* missense mutation that changed threonine-99 to asparagine-99 (Ume6p-T99N) (Bowdish *et al.*, 1995). Surprisingly, in contrast to other *ume6* mutations, the *rim16-12* mutant was still able to repress transcription of a heterologous reporter construct containing the URS1 element, but had a defect in the transcriptional activation of early meiotic genes in sporulation media (Bowdish *et al.*, 1995).

Two models for *UME6* regulation of early meiotic gene expression were then tested. In the first model, Ume6p is converted from a repressor to an activator by *IME1*. In the second model, Ume6p is strictly a repressor that competes with an activator for binding to the URS1. In order to test these models, wild type Ume6p and Ume6p-T99N were fused to a LexA DNA binding domain and tested for their ability to activate transcription of a *GAL1-lacZ* fusion gene. Previous experiments had demonstrated that LexA-Ume6p could complement both a *ume6Δ* mutant allele and the *rim16-12* allele, while LexA-Ume6p-T99N could only complement the *ume6Δ* mutant allele (Bowdish *et al.*, 1995). The ability of each LexA fusion to activate transcription was also tested in the presence or absence of Ime1p. Only the wild type LexA-Ume6p fusion was able to activate transcription, and this activation was dependent on the presence of Ime1p, indicating that the *rim16-12* (Ume6p-T99N) mutation

renders the resulting protein either unable to interact with Ime1p or unable to be modified by Ime1p (Bowdish *et al.*, 1995).

The hypothesis that Ume6p is an Ime1p-dependent transcriptional activator predicts that the Ume6p binding site, the URS1 element, should be an Ime1p-dependent UAS. This prediction was tested using an Ime1p-dependent UAS from the *IME2* gene, which consists of two functional elements: a URS1 site and a T_4C site (Bowdish and Mitchell, 1993). These elements were fused separately and together to a heterologous *CYC1-lacZ* reporter gene and assayed in the presence and absence of Ime1p. The results of this experiment demonstrate that the URS1^{IME2} site transmits an Ime1p-dependent activation signal that is increased by the presence of the T_4C site which by itself is unresponsive to Ime1p (Bowdish *et al.*, 1995). Furthermore, the same experiment using the URS1^{CAR1} demonstrated that it could also transmit an Ime1p-dependent activation signal (Bowdish *et al.*, 1995).

Recent experiments have clarified the role of *UME6* in control of meiosis. For these experiments, a diploid strain homozygous for a *ume6Δ* deletion allele was first sporulated and compared to an isogenic wild type strain. At 30°C, the *ume6Δ/ume6Δ* diploid produces approximately 5% asci compared to the 80% seen in the wild type strain (Steber and Esposito, 1995). In order to determine the nature of the sporulation defect, landmark features of meiosis were compared between the *ume6Δ* strain and an isogenic wild type strain. While premeiotic DNA synthesis was unaffected in the *ume6Δ* mutant strain, in the majority of cells tested, meiotic recombination failed to occur, resulting in eventual cell death (Steber and Esposito,

1995). A minor population of cells proceeded beyond this point, presumably due to the action of a *UME6*-independent pathway. DAPI staining of nuclei reveals that less than 5% of cells complete meiosis I and meiosis II, and light microscopy indicates that after 48 hours only 8% of the cells actually form asci, indicating the importance of *UME6* for these processes (Steber and Esposito, 1995). To determine if these meiotic defects correlate with altered gene expression, representative meiosis-specific transcripts were quantitated during sporulation. Unlike the URS1-containing meiotic genes, the vegetative levels of *IME1* transcript remains repressed in *ume6* mutants during growth in glucose (Strich *et al.*, 1994) or acetate (Steber and Esposito, 1995). However, during sporulation the *IME1* transcript accumulates to 3-fold higher levels in the *ume6*Δ strain and fails to decrease after 10 hours, demonstrating that *ume6*Δ is required for reestablishment of *IME1* repression during meiosis (Steber and Esposito, 1995). In accordance with earlier studies, *UME6* was shown to be important for the vegetative repression of early meiotic genes such as *SPO11* and *SPO13*, the meiotic induction of these genes, and the reestablishment of repression (Steber and Esposito, 1995).

Surprisingly, *UME6* is required for the proper timing of mid/late meiotic gene expression. Expression of the middle genes *SPS2* and *SPO12*, which are normally induced approximately 8-10 hours after induction of meiosis, was delayed approximately 6 hours and the expression levels finally achieved were only 65% of those seen in the wild type strain (Steber and Esposito, 1995). A late gene was more severely affected. Expression of *DIT1* normally is

expressed at maximum levels after 12 hours: in the *ume6*Δ strain, it only reached 12% of wild type expression levels after 48 hours (Steber and Esposito, 1995).

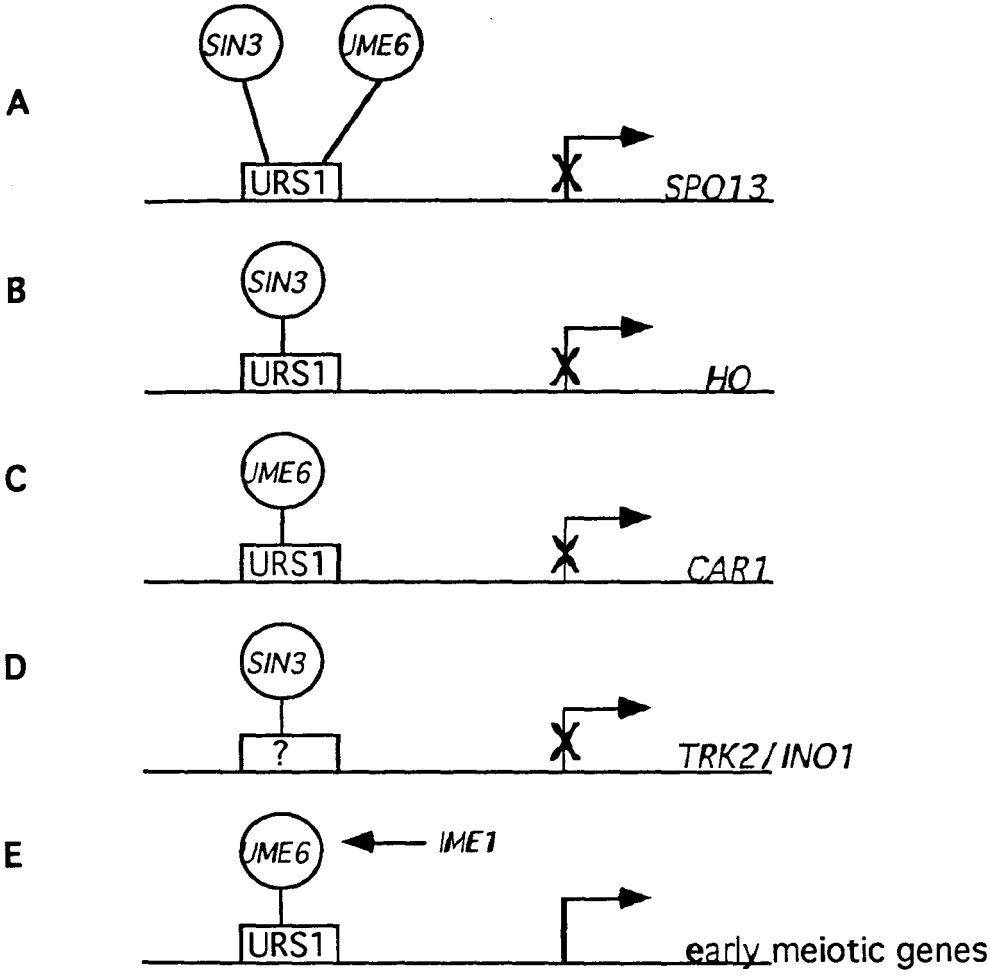
Based on the studies of *UME6* function, the following model for *UME6* regulation of meiosis has emerged: during vegetative growth, *UME6* mediates early meiotic gene repression by both cell-type and nutritional controls through the URS1 element; during meiosis, *IME1* is activated by a loss of cell-type repression through inactivation of *RME1*, and by glucose and nitrogen starvation; this results in the conversion of Ume6p from a repressor to an activator through interaction (direct or indirect) with *IME1*; the activator Ume6p then is able to activate transcription through the URS1 element with support from nearby promoter elements; and finally, this process of activation may be required for the production of regulators needed to reestablish repression.

Current models of *SIN3* and *UME6* function

To summarize, five different systems of regulation using the *SIN3* and *UME6* regulatory genes and the URS1 element can be defined. There are systems that use both *SIN3* and *UME6* as repressors through a URS1-dependent pathway, such as *SPO13* and other early meiotic genes (Fig.3A) (Strich *et al.*, 1989; Strich *et al.*, 1994). There are systems that use either *SIN3* (*e.g.*, *HO*) (Wang *et al.*, 1990) or *UME6* (*e.g.*, *CAR1*) (Park *et al.*, 1992) through URS1-dependent pathways (Fig. 3B,C). There are also systems that use *SIN3* as a repressor but are URS1-independent (*e.g.*, *TRK2* and *INO1*) (Vidal *et al.*, 1995; Slekar and Henry, 1995) (Fig. 3D). Finally, in the

case of the early meiotic genes, *UME6* is required for *IME1*-dependent transcriptional activation mediated by the URS1 element (Bowdish and Mitchell, 1995) (**Fig. 3E**).

Figure 3. Models of *SIN3*, *UME6*, and URS1-mediated regulation. For a complete description of the models refer to text.


A
SIN3
UME6
URS1
SPO13
B
SIN3
URS1
HO
C
UME6
URS1
CAR1
D
SIN3
?
TRK2/INO1
E
UME6
IME1
URS1
early meiotic genes

CHAPTER III
MATERIALS AND METHODS

I. General Methods

Bacteria strains and growth conditions

Escherichia coli DH5α cells [F- *end*A1 *hsd*R17(rK-, mK+) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 Δ(*arg*F-*lacz*ya)U169 ø80d*lacZ* ΔM15 λ-] were cultured in LB medium (1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) supplemented with 50μg/ml ampicillin (LB-amp) for the propagation of plasmids. Solid media contained 2% agar. In order to detect recombinant colonies, indicator plates containing 50μl of 2% (w/v) X-gal [(5-bromo-4-chloro-3-indolyl-β-D-galactoside) in N,N-dimethylformamide] were used. All bacterial strains were grown at 37°C and stored at 4°C for short term storage or frozen at -80°C for long term storage. Transformation competent DH5α bacterial cells (Gibco-BRL, Gaithersburg, MD) were transformed by the $CaCl_2$ method (Sambrook *et al.*, 1989).

Yeast strains and growth conditions

The genotypes and sources of *Saccharomyces cerevisiae* strains used in this study are listed in Table 1.

Table 1. Yeast Strains

Strain	Genotype	Source
BRS1001	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1*	This lab
BRS2005	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, opi1::LEU2*	This study
BRS2009	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, ume6::LEU2*	This study
BRS2011	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ino2::TRP1, ura3::pGAL1-INO2::URA3*	This lab
BRS2013	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ume6::LEU2, ura3::pGAL1-INO2::URA3 ino2::TRP1*	This study
BRS1005	MAT a, *ade1, ino1-13* MAT α, *ade1, ino1-13*	This lab
SFY59	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, ade6*	C. Steber and R.E. Esposito
REE2276	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, ade6, ime1::URA3*	C. Steber and R.E. Esposito
BPA101	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2::URA3*	This lab
BPA102	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO4::URA3*	This lab
BPA103	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-promoterless-cat::URA3*	This lab
JCJ101	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO1::URA3*	This lab
JCJ102	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO1::URA3, ume6::LEU2*	This study
JCJ103	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-MURS::URA3*	This study
JCJ104	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-MURS::URA3, ume6::LEU2*	This study

JCJ105	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2::URA3, ume6::LEU2*	This study
JCJ106	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO4::URA3, ume6::LEU2*	This study
JCJ107	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-1::URA3 ume6::LEU2*	This study
JCJ108	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-2::URA3 ume6::LEU2*	This study
JCJ109	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-5::URA3 ume6::LEU2*	This study
JCJ110	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-6::URA3 ume6::LEU2*	This study
JCJ111	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-6/4::URA3 ume6::LEU2*	This study
JCJ112	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-8/2::URA3 ume6::LEU2*	This study
BPA201	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-1::URA3*	This lab
BPA202	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-2::URA3*	This lab
BPA205	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-5::URA3*	This lab
BPA206	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-6::URA3*	This lab
BPA210	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-6/4::URA3*	This lab
BPA212	MATa, *ade2-1, his3-11,15, leu2-3,112, can1-100, trp1-1, ura3-1, gal4::pBM-INO2-8/2::URA3*	This lab

All yeast strains were maintained at 30°C on YEPD plates (1% yeast extract, 2% Bacto-peptone, 2% glucose and 2% agar) and stored at 4°C. Yeast transformants were selected on synthetic complete media (2% glucose, 0.67% yeast nitrogen base without amino acids [Difco Laboratories], lysine (230mg/L), arginine (20mg/L), leucine (60 mg/L), methionine (20mg/L), threonine (0.3g/L), tryptophan (20 mg/L), adenine (20mg/L), histidine (20mg/L), uracil (20mg/L), and 2% agar) lacking the appropriate nutrient. Where appropriate, 75μM inositol and 1mM choline were added to the media, and 2% (w/v) galactose substituted for glucose.

II. Molecular Methods

Isolation of DNA restriction fragments from agarose gels

Restriction digests were performed according to specifications of the supplier, and the products were fractionated by electrophoresis through 1% agarose in 1X TBE buffer (90mM Tris, 90 mM boric Acid, and 2mM EDTA). DNA fragments were isolated from agarose gels by excision with a razor blade of the appropriate ethidium bromide-stained band. The gel slice was crushed through 1 cc syringe into a 0.45 μm MC Millipore filter unit and frozen on dry ice for one hour or overnight at -80°C. The filter unit containing the gel slice was incubated for 5 minutes at 37°C and centrifuged for 15 minutes at 13000xg. One tenth volume of 3M NaOAc and 3 volumes of 100% ethanol was added to the eluant. The mixture was incubated on dry ice for at least one hour or overnight at -80°C. DNA was precipitated by centrifugation (15 minutes, 13000xg), the DNA pellet

washed with 70% ethanol, and resuspended in sterile distilled H_2O (sdH_2O).

Ligations

Fragments of DNA were joined with linearized vector DNA to form circular plasmids by DNA ligase from bacteriophage T4 (Gibco-BRL). Ligation reactions (20 µl final volume) contained fragment DNA and vector DNA (5:1 molar ratio), 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, and 1 unit of T4 DNA ligase. The reactions were incubated overnight at 15°C, and stopped by heat inactivation at 65°C for 15 minutes.

Plasmid minipreparation

To screen for recombinant DNA products, plasmid DNA was isolated from *E. coli* using an alkaline lysis method. An overnight culture (2ml) grown in LB-amp was pelleted (30 seconds at 13000xg). The cell pellet was resuspended in 250µl of a solution containing 100µg/ml RNase A, 50mM Tris-HCl pH 8.0, 10mM EDTA. To this was added 250 µl of 0.2M NaOH/1% SDS, and the mixture incubated for 5 minutes at room temperature. Subsequently, 250 µl of 2.55M KAc pH 4.8 was added, and the mixture centrifuged for 15 minutes at 13000xg/4°C. The supernatant was transferred to a sterile 1.5ml microfuge tube, and the plasmid DNA precipitated by the addition of 0.6 volumes of isopropanol. Following a one hour incubation on dry ice (-80°C), the plasmid DNA was precipitated by centrifugation (15 minutes at 13000Xg/4°C). The DNA pellet was

washed with 70% ethanol, and the pellet resuspended in 50µl of sdH_2O.

Large scale plasmid isolation

Large scale isolation of plasmid DNA from *E. coli* was performed by an alkaline lysis method. A 50ml bacterial culture grown overnight in LB-amp media at 37°C was pelleted (5 minutes at 3000xg/4°C). The cell pellet was washed once in 10 ml of saline solution (100mM NaCl, 10mM EDTA, 50mM Tris-HCl pH 8.0) and resuspended in 2.4 ml of freshly prepared lysozyme solution (2mg/ml lysozyme in 25mM Tris-HCl pH 7.5, 10mM EDTA, 10mM sucrose) and incubated on ice for 20 minutes. Following the addition of 4.8 ml of a 1% SDS/0.2M NaOH solution, the tubes were mixed gently by inverting and then incubated on ice for 10 minutes. Next, 3 ml of 3M sodium acetate pH 4.6 was added, and the tubes mixed by inversion and incubated on ice for 10 minutes. Cellular debris were pelleted by centrifugation (15 minutes at 48000xg/4°C). The supernatant was collected and treated with 5µl of 10mg/ml RNase A for 15 minutes at 37°C. The samples were extracted once with 10.3 ml of phenol:chloroform (1:1), and the plasmid DNA was precipitated by centrifugation following addition of 2 volumes of cold 100% ethanol (stored at -20°C). The pellet was resuspended in 525µl of sdH_2O and transferred to a sterile 1.5 ml microcentrifuge tube. The plasmid DNA was reprecipitated by adding 100µl of 5M NaCl and 625µl of 13% PEG (6000-8000) and pelleted by centrifugation (10 minutes at 12000xg/4°C). The pellet was washed with 70% ethanol and resuspended in 200µl of sdH_2O. Plasmid DNA concentration was

determined by measuring optical density at 260nm (1 O.D. = 50μg/ml).

Plasmid construction

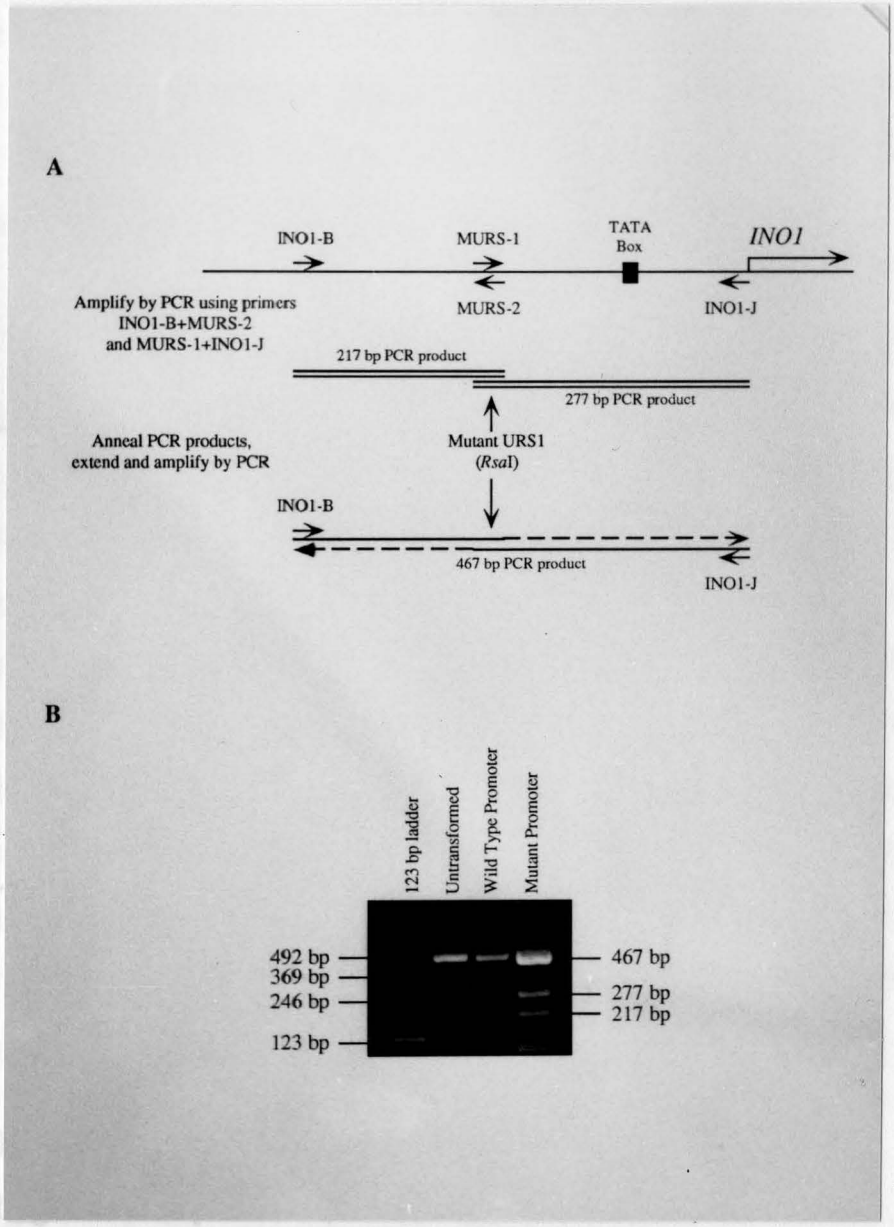
Plasmids pBM-INO2, pBM-INO4, and pBM-INO1 contained PCR-generated promoter sequences fused upstream of the *cat* reporter gene, and have been described in detail elsewhere (Ashburner and Lopes, 1995). Plasmid pBM-MURS contained the portion of the *INO1* promoter found in pBM-INO1 (-453 to +1) with a PCR-generated mutant URS1 element replacing the native URS1 element. The mutant URS1 element was constructed using a previously described strategy (Higuchi *et al.*, 1991) (Fig. 4A). Complimentary oligonucleotides, MURS1 (5' CTTCGTACGCTAAATGCGGC 3') and MURS2 (5' TTAGCGTACGAAGCGC ATAC 3'), containing the desired mutation (*Rsa*I site) (underlined) in the URS1 element (bold) were synthesized. These were used in separate PCR reactions to generate PCR products that overlapped at the mutated URS1 element. These PCR products were purified using the Wizard PCR Prep kit from Promega (Madison, WI), annealed, extended by *Taq* polymerase, and the resulting full-length promoter element was amplified using flanking oligos INO1-B (5' GGGATCCCGGCCGTACTTAGTG 3') and INO1-J (5' GAGATCTTGTTACTTCTTTTTCAC 3'). Creation of the mutation in the URS1 element was verified by digestion with *Rsa*I. The mutated URS1 PCR product was cloned into the pGEM®-T vector (Promega, Madison, WI) to create pGEM-MURS. A *Bam*HI/*Bgl*II restriction fragment containing the *INO1* promoter with the mutant URS1 element was cloned into the *Bam*HI site of pBM2015 (Griggs and

Johnston, 1991) creating pBM-MURS. Plasmid pBM-MURS was digested with *Cla*I and *Sst*II which liberated a fragment containing *GAL4* sequences flanking the promoter-*cat* fusion and the *URA3* selectable marker. Strains BRS1001 (wild-type) and BRS2009 (*ume6*Δ) were transformed with this restriction fragment and uracil prototrophs were selected. Southern blot analysis confirmed integration of the reporter fusions at the *GAL4* locus in single copy. The presence of the *Rsa*I site (*i.e.*, mutant URS1 element) was confirmed by isolating genomic DNA from the transformed strains, amplifying the *INO1* promoter region by PCR, and digesting the resulting PCR product with *Rsa*I. The amplified *INO1* promoter region was not digested by *Rsa*I in either the untransformed strains or the strains that contained the integrated wild-type *INO1* promoter-*cat* fusion, (Fig. 4B). Contrastingly, when DNA from the strains that contained the integrated mutant URS1 was used, three bands were observed after digestion with *Rsa*I (Fig. 4B). The larger band corresponded to the native *INO1* promoter, and the two smaller bands indicated the presence of the mutation in the URS1 element in the promoter-*cat* fusion at the *GAL4* locus.

Generation of yeast strains "knocked out" for *UME6* and *OPI1*

Yeast strains used in this study containing a null allele of the *UME6* gene (*ume6::LEU2*) were generated by transforming the appropriate wild type strain (BRS1001 or BRS2011) with a *Pst*I/*Sst*I restriction fragment from pPL5914 (Strich *et al.*, 1994) (Fig. 5). This fragment contained the *LEU2* selectable marker flanked by

Figure 4. Construction of an *INO1* promoter fragment containing a mutant URS1 element. (A) Schematic depicting PCR mutagenesis of the *INO1* promoter. Two PCR fragments were generated which overlapped in the URS1 region. These PCR products were annealed, extended, and the full length *INO1* promoter fragment containing the mutated URS1 element was amplified by PCR using flanking primers INO1-B and INO1-J. The full-length PCR fragment was inserted upstream of the *cat* reporter gene as described previously (20). (B) Confirmation of the URS1 mutation in the pBM-MURS-*cat* reporter construct after integration into the yeast genome. Genomic DNA was isolated from an untransformed strain (BRS1001), a transformant containing an integrated wild-type *INO1* promoter-*cat* fusion, and a transformant containing an integrated pBM-MURS-*cat* fusion. The genomic DNA was used to amplify the region of the *INO1* promoter using the INO1-B and INO1-J primers. The resulting PCR products were purified and digested with *Rsa*I to confirm the presence of the mutated URS1 element. For reference is shown a 123bp DNA ladder. The 467bp band in the mutant promoter lane results from the native *INO1* promoter which contains a wild-type URS1 element and therefore lacks the *Rsa*I site.


A
INO1-B
MURS-1
TATA Box
INO1
MURS-2
INO1-J
Amplify by PCR using primers INO1-B+MURS-2 and MURS-1+INO1-J
217 bp PCR product
277 bp PCR product
Anneal PCR products, extend and amplify by PCR
Mutant URS1 (RsaI)
INO1-B
467 bp PCR product
INO1-J
B
123 bp ladder
Untransformed
Wild Type Promoter
Mutant Promoter
492 bp
369 bp
246 bp
123 bp
467 bp
277 bp
217 bp

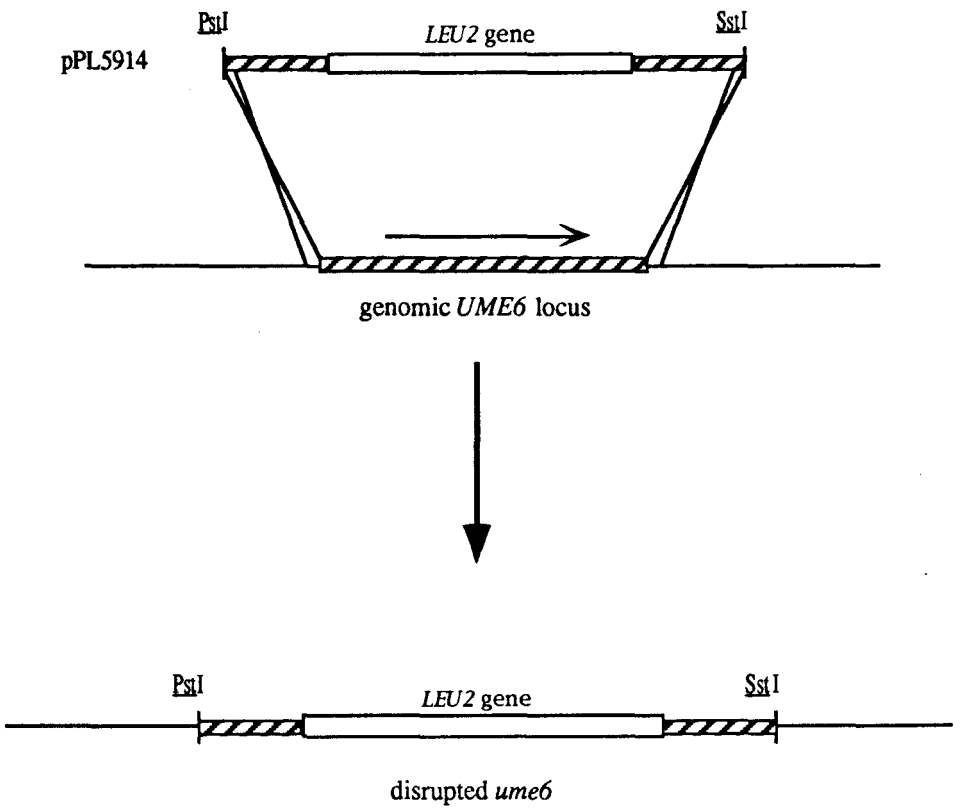


Figure 5. Disruption of the *UME6* gene in *S. cerevisiae*. A *Pst*I/*Sst*I restriction fragment from pPL5914 (Strich *et al.*, 1994) was used to transform a leucine auxotrophic yeast strain, yielding a strain containing a disrupted *ume6* allele.

sequences of the *UME6* gene. Since the ends of DNA fragments are highly recombinogenic (Orr-Weaver *et al.*, 1981), Leu$^+$ transformants arise by recombination between sequences in *UME6*. The Opi$^+$ test was used to confirm proper integration (described below). The yeast strain used in this study containing a null allele of the *OPI1* gene (*opi1::LEU2*) was generated by transforming wild type strain BRS1001 with a restriction fragment containing the *opi1*Δ null allele as previously described (White *et al.*, 1991).

Yeast transformations

Yeast transformations were performed using the one-step transformation of yeast protocol (Chen *et al.*, 1992). The strain to be transformed was grown overnight in YEPD at 30°C. One ml of cells (approximately 2.5 X 10^8 cells) was transferred to a sterile 1.5ml microfuge tube and pelleted for 5 minutes at 3000xg. The pellet was resuspended in 100µl of freshly made transformation buffer (0.2N lithium acetate, 40% PEG 3350, and 100mM DTT) and 50ng-1µg of transforming DNA and 50µg of salmon sperm DNA was added. The tubes were then vortexed and incubated at 45°C for at least one hour. The entire contents of the tubes (cells and buffer) were then spread on selective or "drop out" plates and incubated at 30°C for 4-5 days to obtain transformants.

Preparation of yeast genomic DNA

Yeast genomic DNA was prepared from cultures (5 ml) grown to saturation in YEPD at 30°C (Hoffman and Winston, 1987). The cells were pelleted by centrifugation (10 minutes/1300xg). The cell pellet

was resuspended in 0.5 ml of sdH_2O, and transferred to a sterile 1.5 ml microfuge tube. The cells were pelleted again (5 seconds/13000xg), resuspended in 0.2 ml of lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA) and 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The cells were disrupted by vortexing for 3-4 minutes in the presence of 0.5 ml glass beads (0.45mm diameter), 0.2 ml of 1x TE-8 was added and the mixture centrifuged (5 minutes/13000xg). The supernatant was removed and 1 ml of 100% cold ethanol was added to precipitate the DNA. The DNA was pelleted by centrifugation (5 minutes/13000xg), resuspended in 0.4 ml of 1x TE-8, 3 µl of 10mg/ml RNase A was added, and the mixture incubated for 20 minutes at 37°C. After the incubation, 10 µl of 4M NH_4OAc and 1 ml of cold 100% ethanol were added. The DNA was pelleted by centrifugation (5 minutes/13000xg), and the pellet resuspended in 50 µl of sdH_2O. Typically, 10 µl of genomic DNA was used for restriction digests or PCR amplifications.

RNA analysis by northern and slot blot hybridizations

A. Isolation of total cellular RNA

Total RNA was isolated from yeast strains by the glass bead disruption and hot phenol extraction method of Elion and Warner (1984). Twenty-five ml cultures were harvested at mid-log phase (between 60-80 Klett units, Klett-Summerson colorimeter). Cells were pelleted by centrifugation (5 minutes/3000xg/4°C) and washed once with ice-cold sdH_2O, repelleted and resuspended in 0.5 ml of

LET-1% SDS (0.1M LiCl, 10mM EDTA, 0.01M Tris-HCl pH 7.5, 1%SDS). This suspension was transferred to a sterile 15 ml disposable glass screw cap tube and frozen overnight at -80°C. The following day, the cell suspension was thawed on ice. Once thawed, a 0.5ml volume of glass beads (0.45mm diameter) was added to the cell suspension, and the cells were vortexed for 25 seconds. One hundred µl of phenol/chloroform (1:1) was then added to each sample. The samples were then vortexed 4 x 25 seconds, being placed on ice between each pulse of vortexing. Two ml of LET-0.2% SDS was added to each sample. Two successive hot phenol extractions were then performed by transferring the mixture into a tube containing 2.5 ml of phenol pre-heated to 65°C in a water bath. Each sample was mixed by vortexing for 5 seconds, placed on ice for 4 minutes, and the aqueous layer recovered following centrifugation (10 minutes/1500xg). The final aqueous fraction was transferred to a sterile 15 ml Corex tube. A 3M LiCl solution was added to a final concentration of 0.3M LiCl and the RNA was precipitated overnight at -20°C following addition of 2.5 volumes cold 100% ethanol. The RNA was pelleted by centrifugation (25 minutes/12000xg/4°C) and each pellet was dissolved in 0.4 ml of sdH_2O. The RNA was then reprecipitated by addition of 0.1 ml 0.5M NaCl and 1 ml of cold 100% ethanol. The RNA was pelleted by centrifugation (25 minutes/12000xg/4°C) and resuspended in 200 µl of sdH_2O. The concentration of RNA was determined by measuring the optical density at 260nm (1 O.D. = 40µg/ml).

B. Northern blot hybridization analysis

Ten µg of total RNA was dissolved in sample buffer (20µl total volume)(Sambrook *et al.*, 1989) and heated to 65°C for 5 minutes. One µl of 1mg/ml ethidium bromide was added to the samples, and the RNA was fractionated on a 1.2% (w/v) agarose, 3% (w/v) formaldehyde, 20mM MOPS/1mM EDTA gel. The running buffer was 20mM MOPS pH 7.4 and 1mM EDTA. RNA was transferred to Magna NT Nytran modified nylon membrane (0.45µm) (Micron Separations Inc.) by capillary transfer overnight in 10x SSC (1.5M NaCl, 0.15M NaCit) and the membrane was baked at 80°C under vacuum for 2 hours.

C. Slot blot hybridization analysis

Slot blots were performed by dissolving 2 or 3 µg of total cellular RNA in 400µl of 20X SSC (3M NaCl, 0.3M NaCit) and applying this mixture to 0.45 µm Magna NT Nytran using a 24 well HYBRI-SLOT™ Manifold (Gibco/BRL). After application, the blots were washed once with 20X SSC and baked at 80°C for 2 hours under vacuum.

D. Synthesis of riboprobes (cRNA)

Single-stranded cRNA probes (riboprobes) were synthesized with the Riboprobe Gemini II Core System (Promega, Madison, WI) according to the specifications of the manufacturer. Linearized plasmids used for riboprobe synthesis were as follows [plasmid/restriction enzyme/RNA polymerase/indicated probe (parentheses)]: pAB309Δ/*Eco*RI/SP6/(*TCM1*)(Hudak *et al.*, 1994); pJH310/*Hind*III/T7/(*INO1*)(Hudak*et al.*,1994); pAS103/*Hind*III/T7/(*CHO1*)(Hudak *et al.*, 1994);

pMH203/*Eco*RI/SP6/(*OPI3*)(Hudak*et al.*, 1994);
pTG109/*Hind*III/T7/(*CHO2*) (Lopes *et al.*, 1991);
pGEM-INO2/*Sal*I/ T7/(*INO2*)(Ashburner and Lopes, 1995b);
pPLg/*BamH*I/SP6/(*ACT1*)(C. Steber and R. Esposito, Univ. of Chicago).

Radioactive labeling of DNA by nick translation

Restriction fragments from plasmids were labeled by nick translation according to the specifications of the supplier (Gibco-BRL). The labeled fragments were purified through a 4% Sephadex-G50 column.

Hybridization of blots and quantitation of autoradiograms

Blots were hybridized in a solution (5 ml) containing 1M NaCl, 10mM $NaPO_4$, 0.1% pyrophosphate, 5X Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin), 50% formamide, 1% SDS, and 1.25mg of salmon sperm DNA. The blots were hybridized at 42°C for nick-translated probes, and at 55°C for cRNA probes. Blots were washed three times at 65°C in 2X SSC/0.1% SDS for 15 minutes each time. Results were visualized by autoradiography and quantitated using either the Betascope 603 Blot Analyzer (Beta-gen, Waltham, MA) or by scanning densitometry (HP Scanning Plus 5.0).

Physical mapping

A filter containing yeast chromosomes resolved by CHEF was supplied by Dr. Mike Fasullo (Loyola University of Chicago, Maywood, IL). The strains used to prepare chromosomes for CHEF have been

previously described (Fasullo *et al.*, 1994). Three λ primary clone grid filters (representing 82% of the yeast genome) were generously provided by Dr. L. Riles (Washington University, St. Louis, MO). Hybridizations were carried out as described earlier. The blots were hybridized with a 700bp *Eco*RI/*Hind*III restriction fragment of plasmid pCS4 (provided by C. Steber, University of Chicago; Strich *et al.*, 1994) containing a fragment of the *UME6* gene.

III. Biochemical Methods

Chloramphenicol acetyltransferase (CAT) assay

CAT assays were performed as previously described (Ashburner and Lopes, 1995). Yeast cultures (5 ml) were grown to mid-logarithmic phase (50-80 Klett units) in the appropriate synthetic medium. Cells were pelleted (5 minutes/13000xg/4°C) and washed with 0.5 ml of cold 0.25M Tris-HCl pH 7.5. Cells were resuspended in 0.2 ml of 0.25M Tris-HCl pH 7.5 and 200 µl of glass beads (0.45mm diameter) were added. Cells were disrupted by vortexing 8 times 20 seconds (with 20 seconds on ice between vortexing). Cellular debris was pelleted by centrifugation (15 minutes/13000xg/4°C) and extracts transferred to a sterile 1.5 ml microfuge tube, and then stored at -80°C. Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad). CAT activities were determined using a phase-extraction method (Seed and Sheen, 1988). Briefly, 10 µg of total cellular protein was assayed in a 100 µl reaction containing 50 µl of 0.25M Tris-HCl pH 7.5, 1 µl of 25mM butyryl coenzyme A, and 1 µl [^{14}C]-chloramphenicol

(54mCi/mol) (Amersham). Each reaction was carried out at 37°C for 60 minutes and stopped by addition of 200 µl of a tetramethylpentadecane:xylene mixture (2:1) and vortexing. The organic and aqueous phases were separated by centrifugation (5 minutes/13000Xg) and 160 µl of the upper (organic) phase counted by liquid scintillation after addition to 4 ml of Bio-Safe II scintillation fluid (Research Products International). The lower phase (80 µl) was dried onto 2.4 cm Whatman GF/C glass microfibre filters, and counted by liquid scintillation as before. Units of CAT activity were defined as cpm measured in the upper (organic) phase and expressed as a percentage of total cpm (% conversion) divided by amount of total cellular protein assayed (mg) and the time of incubation (hour).

Phospholipid composition

Steady-state labeling of phospholipids with [^{32}P] orthophosphate (NEN DuPont) was performed as described previously (Atkinson *et al.*, 1980). Cells (10ml) were grown in the presence of 50µCi (9000 Ci/mmol) [^{32}P] orthophosphate for at least 5 generations and harvested (10 minutes/1500xg) in the late-logarithmic phase of growth (5 X 10^7 cells/ml, counted using a hematocytometer). Labeled cells were suspended in 5 ml of cold 5% trichloroacetic acid and incubated on ice for 20 minutes. Cells were pelleted, the tubes were purged with nitrogen gas, and the cell pellets were frozen overnight at -20°C. After thawing the cells, 1 ml of polar solvent [40% ethanol, 13.9% diethyl ether, 2.8% pyridine, 0.027% ammonium hydroxide, and 0.01% butylated hydroxytoluene in chloroform:methanol (2:1)] was added. Lipids were extracted by

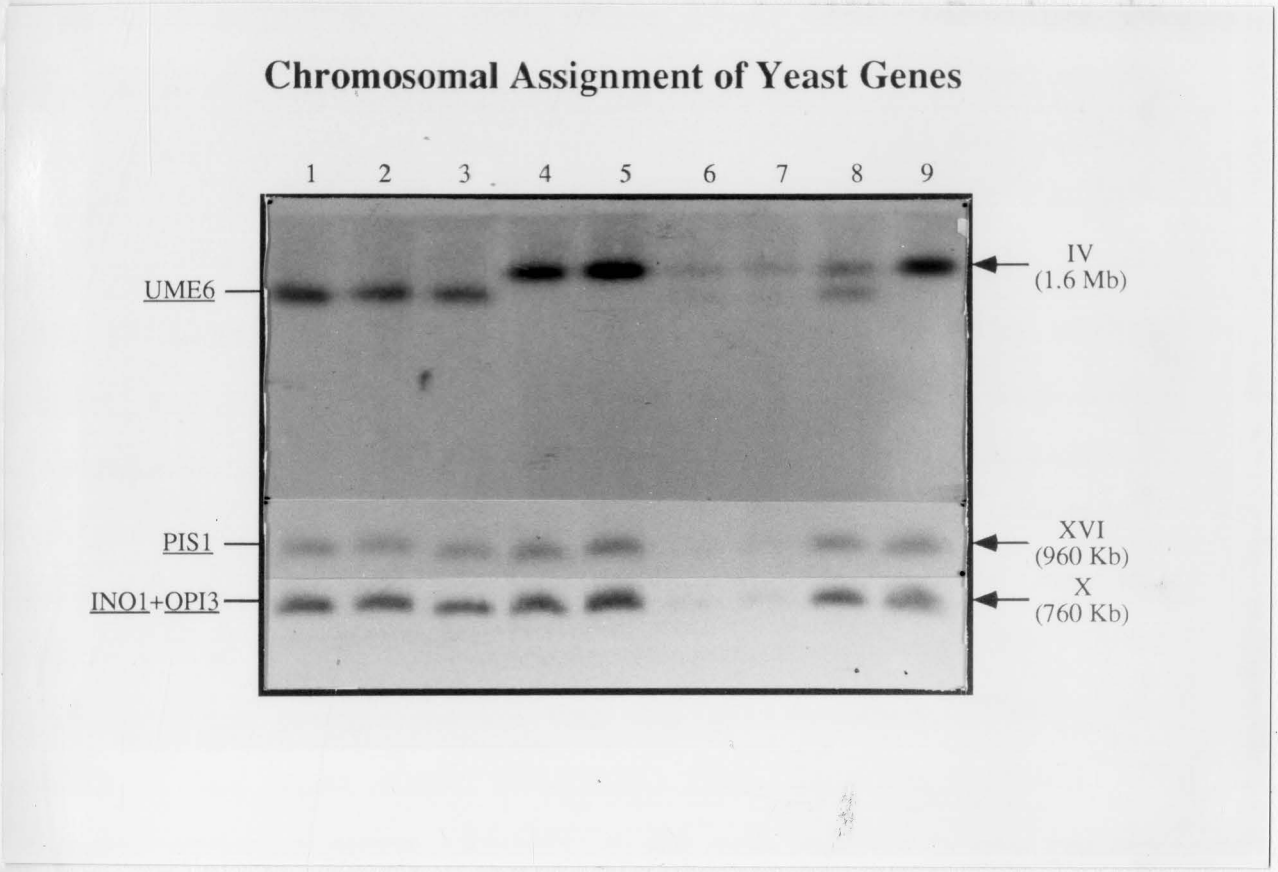
incubation at 60°C for 20 minutes. After cooling to room temperature, 1/2 volume of sdH_20 and 5 volumes of chloroform:methanol (2:1) containing 0.005% butylated hydroxytoluene were added, and the mixture was vortexed (Hanson and Lester, 1980). Solvents were then fractionated by centrifugation (10 minutes/1500xg) and the lower (phospholipid-containing) layer was transferred to a dram vial and dried under a stream of nitrogen gas. The lipid pellet was dissolved in 20 µl of chloroform:methanol (2:1) and separated by two-dimensional paper chromatography after spotting onto chromatography paper (Whatman SG81 treated with 2% EDTA pH 7.4) as described by Steiner and Lester (1972). The solvent for the first dimension was chloroform:methanol:2.8% ammonium hydroxide:sdH_20 (66:27:3:0.8) and the solvent for the second dimension was chloroform:methanol:acetic acid:sdH_20 (32:4:5:1). Phospholipids were visualized by autoradiography and quantitated by liquid scintillation.

CHAPTER IV
RESULTS

Chromosomal location of *UME6*

The *UME6* gene was one of the regulatory genes of phospholipid biosynthesis that remained unmapped genetically or physically. To determine the chromosomal location of the *UME6* gene, I hybridized a Southern blot of yeast chromosomes fractionated by CHEF, with a *UME6*-specific 700bp *EcoR*I/*Hind*III restriction fragment from plasmid pCS4 (Strich *et al.*, 1994). The Southern blot was generously provided by Dr. Mike Fasullo, and the yeast strains used for this experiment are described elsewhere (Fasullo *et al.*, 1994). This experiment localized *UME6* to chromosome IV (Fig. 6). To determine the location of *UME6* on chromosome IV, the *UME6*-specific probe was hybridized to 3 grid filters containing ordered λ clones comprising approximately 82% of the yeast genome (courtesy of Dr. Linda Riles, Washington Univ.). The two overlapping λ clones which were identified localized the *UME6* gene to the right arm of chromosome IV, proximal to the *pet14* gene. This localization is in good agreement with another published report that links *UME6* to *RAD9* which is in close proximity to *pet14* (Strich *et al.*, 1994).

Figure 6. Chromosomal assignment of *UME6*. A Southern blot of yeast chromosomes separated by CHEF. The shifting banding pattern indicated by *UME6* are due to a translocation of chromosome IV onto chromosome II (*CEN*::IV). Lanes 1-3 are a YB100 haploid strain with an induced translocation. Lanes 4,5, and 9 are a YB101 wild type diploid strain. Lanes 6-8 are the YB101 diploid strain with an induced translocation. The CHEF southern was sequentially hybridized with probes specific for the *UME6*, *INO1*, *PIS1*, and *OPI3* genes. (Figure reprinted with permission from Anderson, 1996).


Chromosomal Assignment of Yeast Genes
1
2
3
4
5
6
7
8
9
UME6
PIS1
INO1+OPI3
IV
(1.6 Mb)
XVI
(960 Kb)
X
(760 Kb)

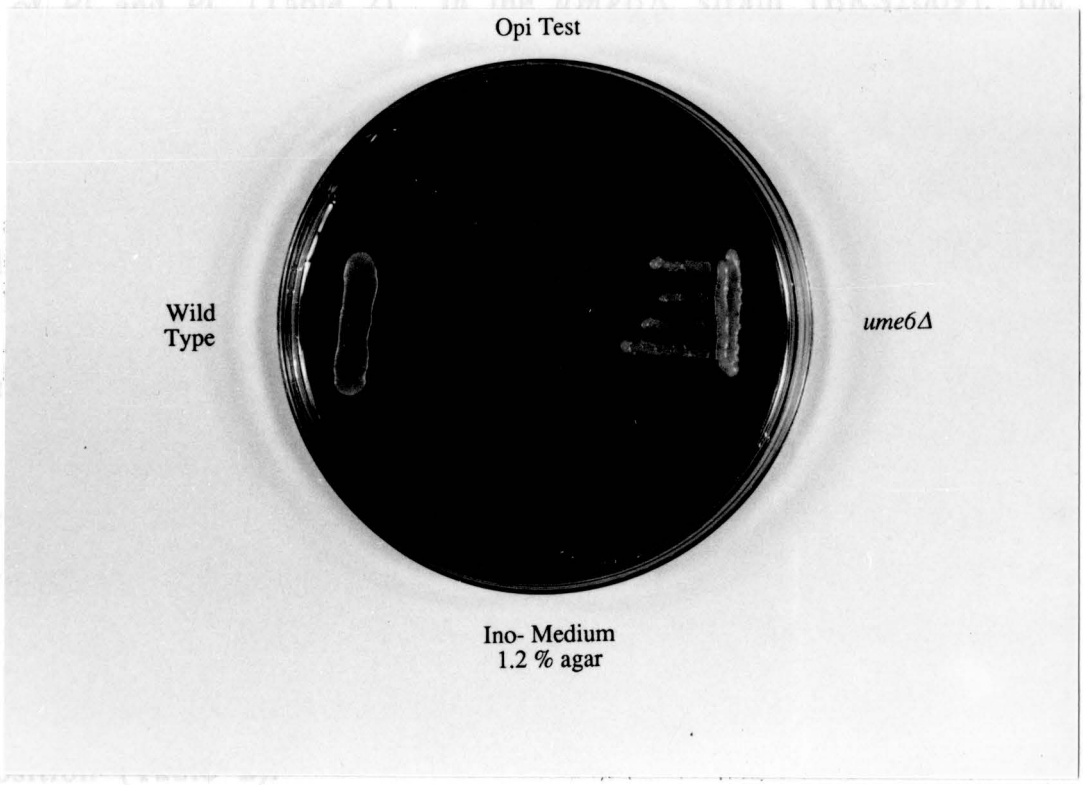
A *ume6Δ* mutant strain had an Opi+ phenotype

One class of regulatory mutants that affect phospholipid biosynthesis share the overproduction of inositol (Opi+) phenotype which is excretion of inositol into the growth media (Greenberg *et al.*, 1982; Hudak, 1994). In the case of the *opi1Δ* and *sin3Δ* mutants, this Opi+ phenotype correlates with the constitutive overexpression of the *INO1* gene (Hudak *et al.*, 1994; White *et al.*, 1991). Based on the presence of the URS1 element in the *INO1* promoter (Lopes *et al.*, 1993), it seemed plausible that *UME6* may be involved in repression of *INO1*. Therefore, a *ume6Δ* mutant strain may also display the Opi+ phenotype. To examine this possibility, a wild-type (BRS1001) and a *ume6Δ* mutant strain (BRS2009) were patched onto media lacking inositol, and allowed to grow at 30° for three days. After three days, a suspension of a diploid tester strain which is an inositol auxotroph (BRS1005) was streaked away from the original patches. The tester strain was expected to grow if inositol had been excreted into the media. This experiment showed that the *ume6Δ* mutant strain (BRS2009) did excrete inositol into the growth media, allowing for growth of the tester strain (BRS1005) (Fig. 7). As expected, the isogenic wild-type strain (BRS1001) did not support growth of the tester strain (BRS1005) (Fig. 7).

Steady-state phospholipid composition is altered in a *ume6Δ* mutant strain

Since an *opi1* mutation leads to an alteration in steady-state phospholipid composition (Klig *et al.*, 1985), I examined the effect of a *ume6Δ* mutation on the phospholipid composition of yeast cellular

Figure 7. The *ume6*Δ mutant has an Opi$^+$ phenotype. Wild-type (BRS1001) and *ume6*Δ (BRS2009) strains were grown on complete synthetic media lacking inositol for 72 hours at 30°C. The inositol auxotroph tester strain (BRS1005) was then streaked away from the patches and inositol cross-feeding was scored after incubation at 30°C for 72 hours.


Opi Test
Wild Type
ume6Δ
Ino- Medium
1.2 % agar

phospholipids. For this, a wild type (BRS1001) and a *ume6*Δ strain (BRS2009) were grown in repressing (I+C+) and derepressing (I-C-) media and labeled with [^{32}P]-orthophosphate. The relative percentages of the cellular phospholipids were then determined (Table 2). Since a *ume6*Δ mutation excretes inositol into the growth media, I expected a dramatic change in the relative levels of the phospholipids. The *ume6*Δ mutation mostly affected the relative levels of PI and PC (Table 2). In the *ume6*Δ strain (BRS2009), the levels of PI under either growth condition are approximately 19%, which is an intermediate level compared to the levels of PI in the wild type strain (BRS1001) (Table 2). In the wild type strain (BRS1001), the levels of PI increase from approximately 15% to 24% when inositol and choline are added to the growth media (Table 2). The *ume6*Δ mutation also had an intermediate effect on the relative levels of PC. In the wild type strain (BRS1001), levels of PC under derepressing conditions (I-C-) are approximately 40%, and the amount of PC falls to approximately 34% under repressing conditions (I+C+) (Table 2). In contrast, the levels of PC in the *ume6*Δ mutant strain (BRS2009) are approximately 37% regardless of media composition (Table 2).

Regulation of phospholipid biosynthetic gene expression was altered in a *ume6*Δ mutant strain

Expression of the phospholipid biosynthetic genes is maximally repressed when cells are grown in the presence of inositol and choline (Nikoloff and Henry, 1991). This repression absolutely

Table 2. Steady State Phospholipid Composition

Strain	Medium	PI	PS	PE	PC
WT	I-C-[a]	14.6	11.5	15.1	40.3
WT	I+C+[b]	23.8	8.9	14.0	33.9
ume6	I-C-[a]	18.8	11.3	18.3	37.8
ume6	I+C+[b]	18.9	7.8	15.8	36.3

a. complete synthetic medium lacking inositol and choline

b. complete synthetic medium supplemented with 75 μM inositol and 1 mM choline

values represent the average of at least two trials

requires the product of the *OPI1* gene. That is, mutations in the *OPI1* gene have been shown to have a pleiotropic effect on repression of the phospholipid biosynthetic genes, as both *INO1* and *CHO1* were constitutively overexpressed in the presence or absence of inositol and choline (Bailis *et al.*, 1987; Hirsch and Henry, 1986). In addition to the *OPI1* gene, the *SIN3* gene is also required to properly regulate expression of the phospholipid biosynthetic genes in response to inositol and choline (Hudak *et al.*, 1994). The pleiotropic phenotype of a *sin3* mutant is surprising because experimental evidence suggests that *SIN3* functions through the URS1 element which is only found in the promoter of the *INO1* gene (Hudak *et al.*, 1994; Lopes *et al.*, 1993). In addition to *SIN3*, *UME6* is also linked to URS1-mediated repression (Park *et al.*, 1992). Since a *sin3*Δ mutation has a pleiotropic effect on phospholipid biosynthetic gene expression and the products of the *UME6* and *SIN3* genes often function collectively, we examined if the *UME6* gene also had a role in controlling expression of the phospholipid biosynthetic genes. For this, total RNA was isolated from wild type strain (BRS1001) and an isogenic *ume6*Δ mutant strain (BRS2009) grown in media lacking (derepressing) or containing inositol and choline (repressing). For comparison, we also isolated RNA from an *opi1*Δ mutant strain (BRS2005). Expression of the phospholipid biosynthetic genes was quantitated by slot blot hybridization with appropriate cRNA probes, and normalized for loading variations to expression of the constitutive *TCM1* gene (Lopes *et al.*, 1991).

Since a strain harbouring a *ume6*Δ allele had the Opi$^+$ phenotype (Fig. 7), I first examined expression of the *INO1* gene

because its overexpression typically correlates with the Opi+ phenotype (White *et al.*, 1991). Quantitation of *INO1* mRNA levels (Fig. 8) in these strain backgrounds demonstrated the different effects the negative regulators *OPI1* and *UME6* had on *INO1* gene expression. As has been shown previously, in the *opi1*Δ mutant background *INO1* was overexpressed in the presence or absence of inositol and choline (Hirsch and Henry, 1986; White *et al.*, 1991) (Fig. 8). Contrastingly, in the *ume6*Δ background, the *INO1* gene was modestly overexpressed in derepressing conditions, and the level of overexpression did not approach the levels demonstrated by the *opi1*Δ mutant strain (Fig. 8).

As has been reported for the *sin3* mutant strain (Hudak *et al.*, 1994), I observed that expression of other phospholipid biosynthetic genes (*CHO1*, *CHO2*, and *OPI3*) was also altered by the *ume6*Δ mutation (Fig. 9). In marked contrast to its effect on *INO1* gene expression, the *ume6*Δ mutation led to a significant decrease in the expression of the other phospholipid genes to wild-type repressed levels (Fig. 9). The *opi1*Δ mutation led to constitutive expression of these same genes at levels greater than or equal to those seen in the wild-type background under derepressing conditions (Fig. 9) which was similar to its effect on *INO1* expression (Fig. 8).

INO2-cat expression was altered in a *ume6*Δ mutant strain

The *ume6*Δ mutation eliminated derepression of *CHO1*, *CHO2*, and *OPI3* gene expression (Figure 9). This raised the possibility that

Figure 8. The *ume6*Δ mutant affects regulation of the *INO1* gene. The amount of *INO1* transcript was determined by counting *INO1*-specific cpm of quantitative slot blots and normalized for loading variations using the constitutively expressed *TCM1* transcript (Lopes *et al.*, 1991). Each value represents the relative level of *INO1* expression from wild-type (BRS1001), *opi1*Δ (BRS2005), or *ume6*Δ (BRS2009) strains grown in complete synthetic media lacking (hatched) or containing (black) 75μM inositol and 1mM choline. Values represent the average of at least 3 independent assays, and standard deviations are indicated.

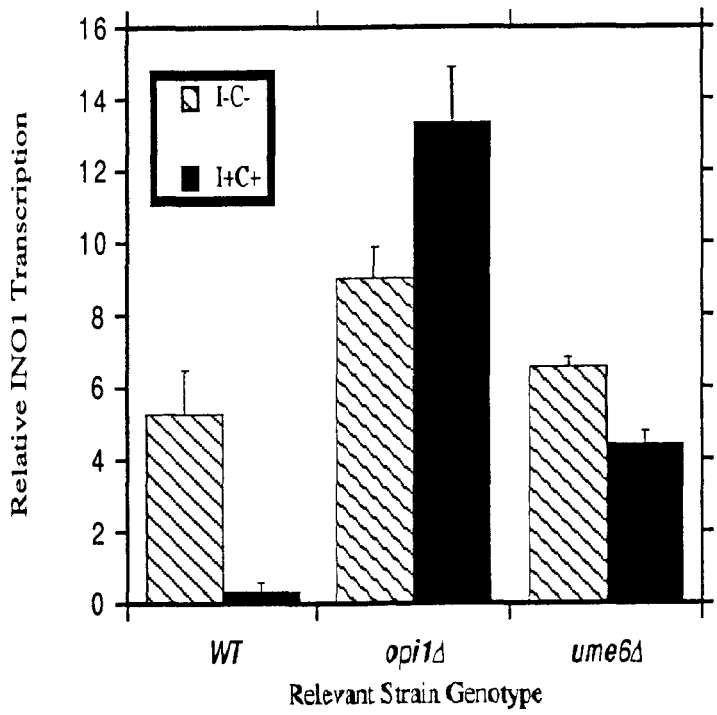
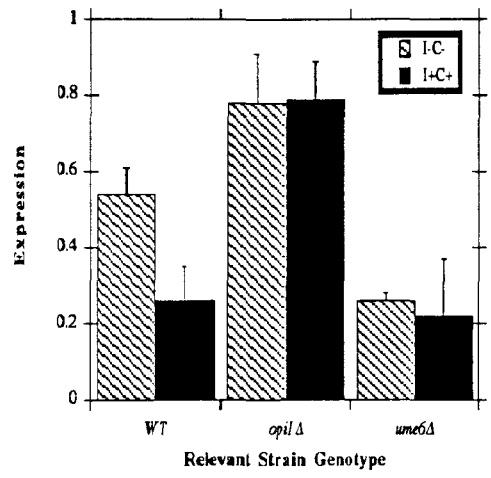
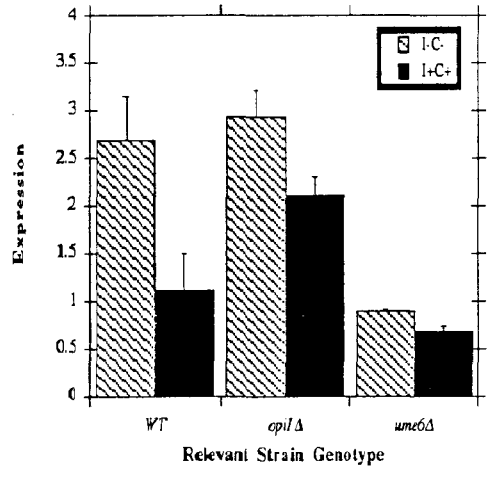

16
14
12
10
8
6
4
2
0
I-C-
I+C+
Relative INO1 Transcription
WT
opi1Δ
ume6Δ
Relevant Strain Genotype

Figure 9. The *ume6*Δ and *opi1*Δ mutations have different effects on transcription of the *CHO1* (A), *CHO2* (B), and *OPI3* (C) genes. The amount of transcript was determined by counting gene-specific cpm of quantitative slot blots and normalized for loading variations using the constitutively expressed *TCM1* transcript (Lopes *et al.*, 1991). Each value represents the relative level of gene expression from wild-type (BRS1001), *opi1*Δ (BRS2005), or *ume6*Δ (BRS2009) strains grown in complete synthetic media lacking (hatched) or containing (black) 75μM inositol and 1mM choline. Values represent the average of at least 3 independent assays, and standard deviations are indicated.

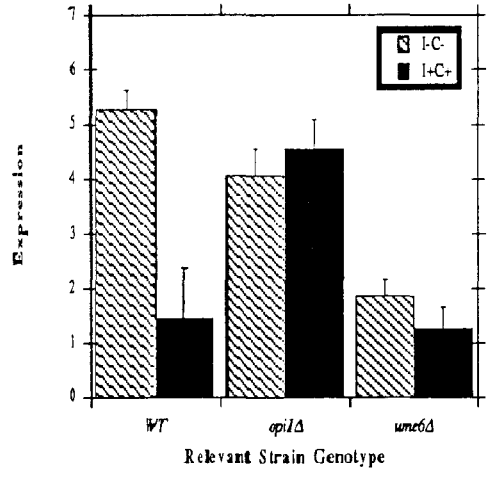
CHO1 Transcription



CHO2 Transcription



OPI3 Transcription

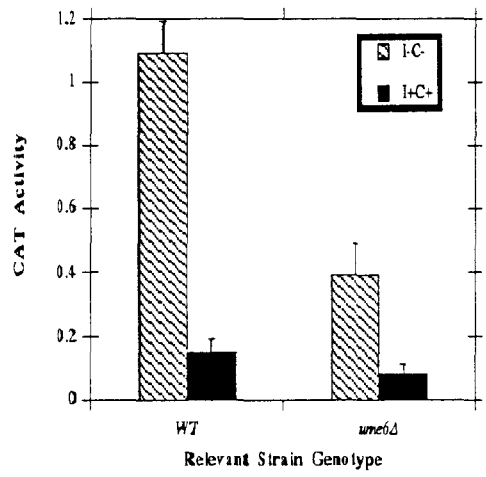


the *ume6*Δ mutation had altered transcription of *INO2* and *INO4* activator genes. Previous work demonstrates that expression of the *INO2* transcriptional activator gene is regulated in the presence of inositol and choline in a manner similar to that of the other phospholipid biosynthetic genes (Ashburner and Lopes, 1995 or 1995b). Based on these findings, I examined expression of an *INO2-cat* gene in the wild-type (BRS1001) and *ume6*Δ mutant (BRS2009) strains under repressing and derepressing conditions. For this, I used a plasmid that contains 500 basepairs of the sequence upstream of the AUG translation start codon of the *INO2* gene fused to a *GAL4-cat* fusion reporter (Ashburner and Lopes, 1995). A single copy of this fusion was integrated into the yeast genome by homologous recombination at the *GAL4* locus. I found that expression of the *INO2-cat* reporter was dramatically reduced in the *ume6*Δ strain (BRS2009) as compared to the isogenic wild-type strain (BRS1001) (Fig. 10A). In the *ume6*Δ strain, CAT activity was reduced approximately 2-fold under repressing conditions, and reduced approximately 3-fold under derepressing conditions (Fig. 10A).

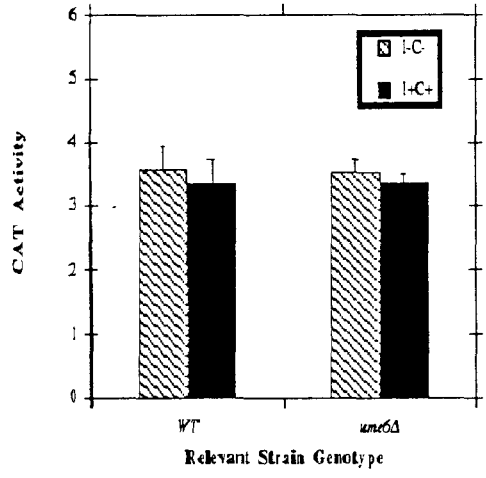
Using the same strategy, I also tested whether expression of the *INO4* positive regulatory gene was altered in the *ume6*Δ strain. Previous work demonstrates that *INO4-cat* is constitutively expressed under both repressing and derepressing conditions (Ashburner and Lopes, 1995). I observed that *INO4-cat* expression was unaffected by the *ume6*Δ mutation (Fig. 10B). Thus, *UME6* is required for proper regulation of *INO2* gene expression. This correlates with the observation that, of the two transcriptional

Figure 10. The *ume6*Δ mutation affects expression of the *INO2-cat* gene (A), but not the *INO4-cat* gene (B). CAT activity in wild-type (BRS1001) and *ume6*Δ (BRS2009) strains grown in complete synthetic media lacking (hatched) or containing (black) 75μM inositol and 1mM choline. Values represent the average of at least 3 independent assays, and standard deviations are indicated.

INO2-cat Expression



INO4-cat Expression



activator genes, only *INO2* expression is regulated in response to inositol and choline (Ashburner and Lopes, 1995).

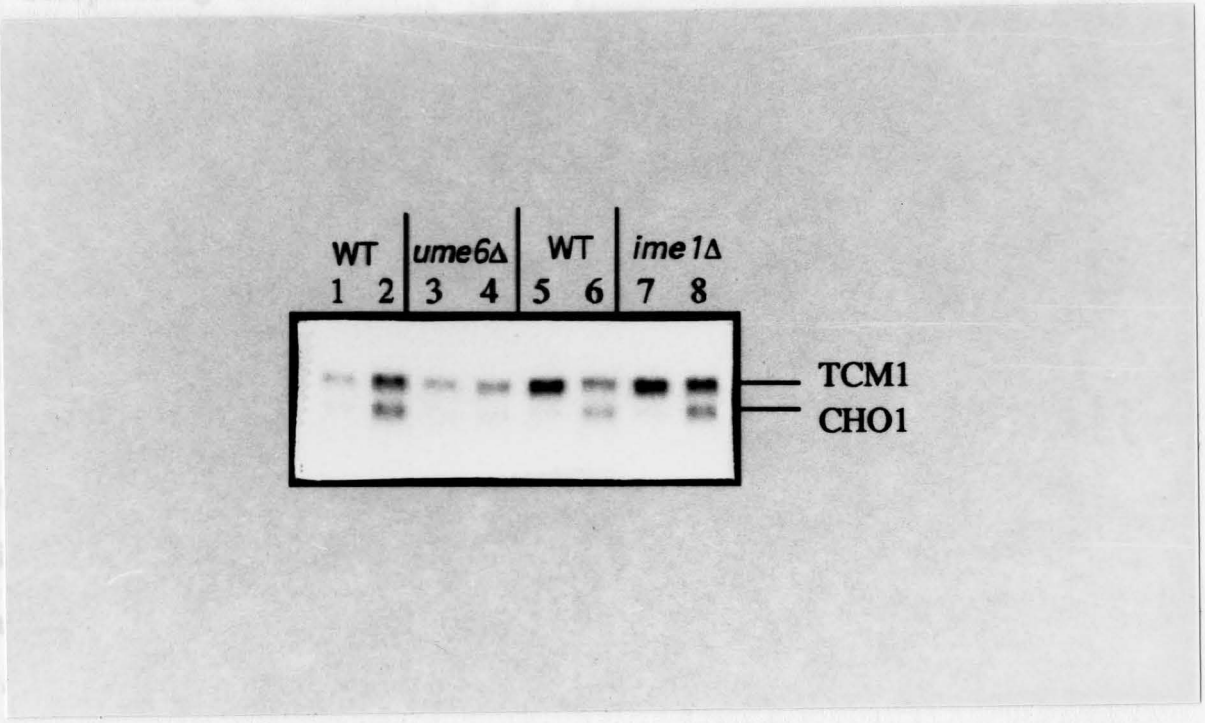
Induction of *CHO1* gene expression is not dependent on the *IME1* gene

The Ume6p-dependent induction of early meiotic genes has been shown to require the *IME1* gene (Bowdish *et al.*, 1995). This raised the possibility that the *IME1* gene might also be required for Ume6p-dependent induction of the *CHO1* gene. To examine this, total RNA was isolated from a wild type strain (SFY59) and an isogenic strain carrying an *ime1*Δ allele (REE2276) and *CHO1* transcription was assessed by Northern blot hybridization. For comparison, *CHO1* transcription was also examined in a second wild type strain (BRS1001) and an isogenic strain harboring a *ume6*Δ allele (BRS2009). The data showed that *CHO1* transcription was unaffected by the *ime1*Δ mutant allele (Fig. 11, compare lanes 5 and 7, and lanes 6 and 8).

***UME6* exerted repression through the URS1 element found in the *INO1* promoter**

Previous work showed the presence of a functional URS1 element in the *INO1* promoter. This URS1 element can function in its native context to repress expression of a heterologous *INO1-CYC1-lacZ* reporter construct (Lopes *et al.*, 1993). In addition, work on the *CAR1* gene demonstrated that the *UME6* gene was required for repression mediated by the URS1 element found in the *CAR1*

Figure 11. Induction of *CHO1* gene expression is *IME1*-independent. Transcription of the *CHO1* gene was assessed by Northern blot hybridization. Hybridization to the constitutively expressed *TCM1* transcript (Lopes *et al.*, 1991) was used as a control for loading variations. The large variations in *TCM1* levels represent errors in sample loading. Total cellular RNA was isolated from a *ume6*Δ mutant strain (BRS2009) (lanes 3 and 4) and an isogenic wild type strain (BRS1001) (lanes 1 and 2). RNA was also isolated from an *ime1*Δ mutant strain (REE2276) (lanes 7 and 8) and an isogenic wild type strain (SFY59) (lanes 5 and 6). Cells were grown in complete synthetic media lacking (even-numbered lanes) or containing (odd-numbered lanes) 75μM inositol and 1mM choline.


WT
ume6Δ
WT
ime1Δ
1 2 3 4 5 6 7 8
TCM1
CHO1

promoter (Luche *et al.*, 1993). These observations prompted me to examine the effect of a *ume6*Δ mutation on expression directed by an *INO1* promoter with a mutant URS1 element. For this analysis, I used wild type (BRS1001) and *ume6*Δ mutant (BRS2009) strains harbouring either a wild type or URS1-mutant *INO1* promoter fused to the *cat* reporter gene. These strains were grown under repressing and derepressing conditions, and CAT activity was assayed.

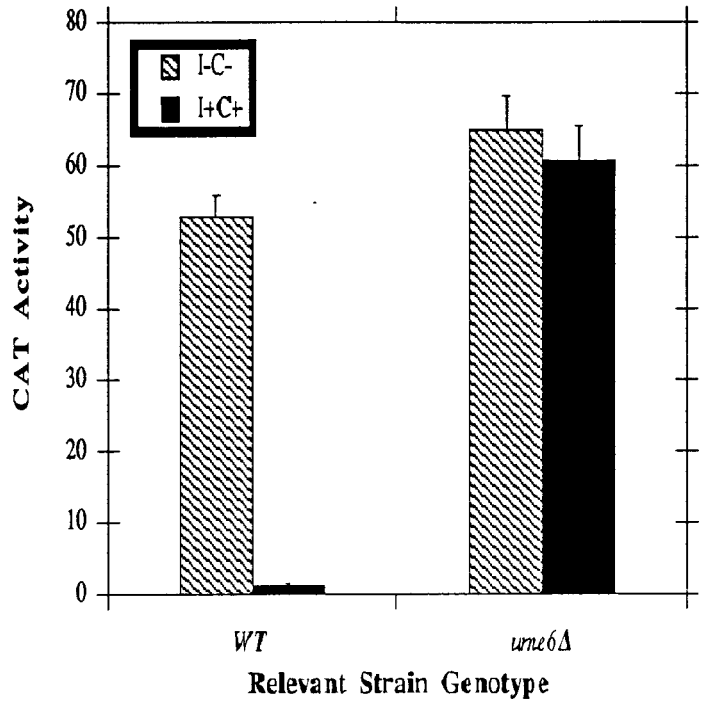
When the *cat* construct containing the native *INO1* promoter was assayed (Fig. 12A), the pattern of regulation in the wild-type (BRS1001) and *ume6*Δ (BRS2009) strains was similar to the regulation of *INO1* transcript levels in these two strains (Fig. 8). That is, the *ume6*Δ mutation caused an increase in expression of the *INO1* gene (Fig. 8) and an increase in CAT activity that was not sensitive to the presence of inositol and choline (Fig. 12A). Mutating the URS1 element in the *INO1* promoter-*cat* fusion also led to constitutive CAT activity in both the wild-type (BRS1001) and *ume6*Δ (BRS2009) strains (Fig. 12B). The lack of synergy between the mutant URS1 and *ume6*Δ mutation, indicated that *UME6* exerted its repression on *INO1* expression through the URS1 element in the *INO1* promoter.

The *INO2* promoter contains a region required for regulation by *UME6*

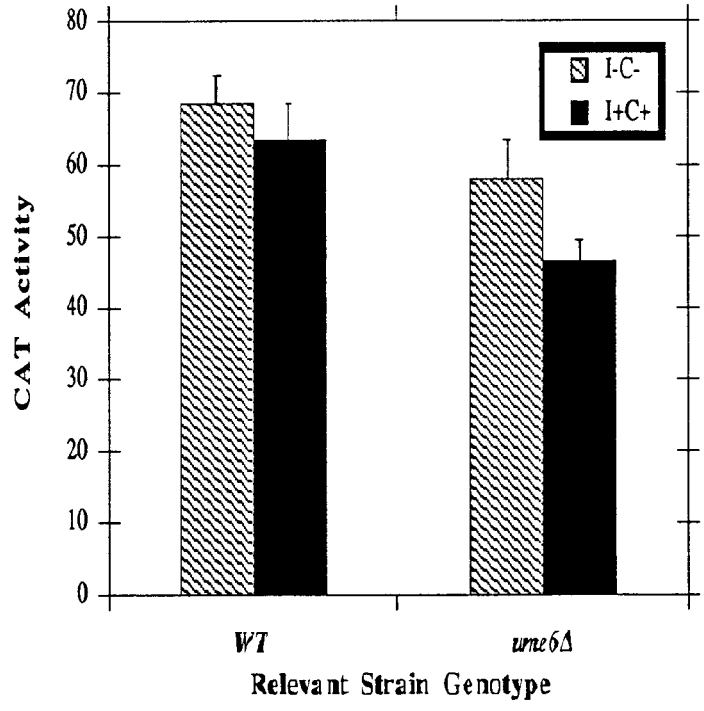
Since expression of an *INO2-cat* reporter construct was reduced relative to wild type in a *ume6*Δ strain (Fig. 10A), I examined deletion constructs of the *INO2* promoter to determine if there was a novel *cis*-acting element that *UME6* works through. In addition, *UME6* can also function as an activator (Bowdish *et al.*, 1995). These

Figure 12. *UME6* represses *INO1* through the URS1 element. The effect of a *ume6Δ* mutant on expression from a wild-type *INO1* promoter (A) and an *INO1* promoter containing a mutant URS1 element (B). CAT activity in wild-type (BRS1001) and *ume6Δ* (BRS2009) strains grown in complete synthetic media lacking (hatched) or containing (black) 75μM inositol and 1mM choline. Values represent the average of at least 3 independent assays, and standard deviations are indicated.

Wild Type *INO1* Promoter



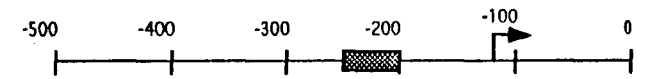
INO1 Promoter With Mutant URS1 Element



observations raised the possibility that *UME6* may work directly at the *INO2* promoter to activate *INO2* expression, despite the lack of any identifiable URS1 element. The deletion constructs that were chosen for analysis had previously been identified as having measurable *INO2-cat* expression in a wild type background (Ashburner, 1995). Constructs number 1 and 2, which respectively delete 100bp and 200bp of the 5' end of the *INO2* promoter, show the same phenotype as the full length *INO2* promoter. That is, the levels of CAT activity in the *ume6*Δ strain are 3-4 fold lower than those seen in the wild type strain (Fig. 13). Two deletions (numbers 5 and 6/4) are the most informative. Construct number 5, which removes only the 3' 50bp of the *INO2* promoter, is essentially unaffected by the *ume6*Δ mutation (Fig. 13). Similarly, internal deletion construct (6/4) which lacks a 50bp region containing the UAS_{INO} element leads to an increase in overall CAT activity in the wild type strain, and remains constitutively elevated in the *ume6*Δ strain (Fig. 13), indicating that these regions may be important for *UME6* regulation of *INO2* (Fig. 13).

Figure 13. Summary of *INO2* promoter deletion analysis. Promoter fragments were fused to the *cat* reporter gene and integrated in single copy at the *GAL4* locus in a wild type (BRS1001) and *ume6*Δ mutant strain (BRS2009). CAT activity values are the average from at least three independent experiments, and standard deviations were less than 20% in all cases. I-C-, complete synthetic medium lacking inositol and choline. I+C+, complete synthetic medium containing 75μM inositol and 1mM choline. UAS, UAS_{INO} element.

INO2 Promoter Analysis



Deletion No.		WT CAT Activity		*ume6Δ* CAT Activity	
		I-C-	I+C+	I-C-	I+C+
Full	UAS	1.16	0.18	0.38	0.06
1		0.91	0.14	0.26	0.07
2		1.08	0.13	0.31	0.05
3		0.01	0.01		
4		0.01	0.01		
5		2.61	1.84	1.65	1.37
6		0.04	0.04	0.07	0.07
7		0.01	0.01		
8		0.01	0.01		
9		0.01	0.01		
6/4		1.85	2.47	1.47	1.42
7/3		0.01	0.01		
8/2		1.77	0.50	0.75	0.13

= Essential Region

= Transcription Initiation Region

UME6 affects *CHO1* expression through regulation of *INO2* levels

In a *ume6*Δ mutant strain (BRS2009), expression of the *CHO1*, *CHO2*, and *OPI3* structural genes is uninducible, and they are constitutively expressed at levels seen in a wild type strain (BRS1001) under repressing conditions (Fig. 9). Expression of the *cat* reporter gene driven by the *INO2* promoter in a *ume6*Δ mutant strain was also markedly reduced in the presence or absence of inositol and choline (Fig. 10A). One effect of decreased *INO2* expression may be a decrease in transcription of its target genes which lack a URS1 element in their promoters, namely the *CHO1*, *CHO2*, and *OPI3* genes. To directly determine the role of *INO2* expression in the regulation and expression of the target genes, I used a system previously shown to uncouple *INO2* expression from the inositol response by placing it under the control of the galactose-inducible *GAL1* promoter (Ashburner and Lopes, 1995b). In this system, a wild type yeast strain was created by integrating the plasmid pGAL1-INO2 into the genome of an *ino2*Δ strain at the *URA3* locus. This ensured all *INO2* expression originated from the *GAL1-INO2* hybrid gene. I used this *GAL1-INO2* containing (BRS2011) to generate an isogenic *ume6*Δ mutant strain (BRS2013). Both wild type (BRS2011) and *ume6*Δ strains (BRS2013) were then grown in media containing either 0.1% galactose or 0.5% galactose and lacking (I-C-) or containing (I+C+) inositol and choline. These concentrations of galactose were chosen based on previous experiments demonstrating a linear relationship between the expression of *INO2* and its target genes between 0.1% and 0.5% galactose (Ashburner

and Lopes, 1995b). Total RNA was isolated and used to determine transcript levels using Northern and slot blot hybridizations. In these experiments, the constitutively expressed *ACT1* gene was used for normalization since *TCM1* gene expression is sensitive to carbon source (J. Warner, personal communication). Expression of *INO2* in the wild type strain (BRS2011) (Ashburner and Lopes, 1995b) and in the *ume6*Δ strain (BRS2013) was shown to be responsive to the amount of galactose in the growth media and not responsive to the presence or absence of inositol and choline (Fig. 14). Therefore in the *ume6*Δ mutant strain (BRS2013), as in the wild type strain (BRS2011), *INO2* expression was uncoupled from the inositol/choline response.

Previous experiments have demonstrated that the *CHO1* gene is still regulated in response to inositol and choline even when *INO2* transcription is not (Ashburner and Lopes, 1995b). The *ume6*Δ mutant strain (BRS2013) allowed me to determine whether underexpression of *INO2* was responsible for the defect in *CHO1* gene expression in a *ume6*Δ mutant strain (see Fig. 9). Total RNA was isolated from the wild type strain (BRS2011) and *ume6*Δ mutant strain (BRS2013) grown in media containing 0.1% or 0.5% galactose and lacking (I-C-) or containing (I+C+) inositol and choline. The RNA was analyzed by Northern and slot blot hybridization to directly quantitate *CHO1* mRNA levels. The Northern blot (Fig. 15) displays the regulation of *CHO1* in response to inositol and choline despite the constitutive expression of the *INO2* gene. Quantitation of *CHO1* mRNA levels (relative to *ACT1* mRNA levels) demonstrate that increasing the concentration of *INO2* in the *ume6*Δ mutant strain

Figure 14. Uncoupling *INO2* expression from the inositol/choline response. Expression of *INO2* transcript from *ume6*Δ mutant strain (BRS2013) grown in complete synthetic media containing 0.1% or 0.5% galactose and either lacking (solid) or containing (hatched) 75μM inositol and 1mM choline. The amount of *INO2*-specific cpm was determined through analysis of slot blot hybridizations and normalized for loading variations to the constitutively expressed *ACT1* transcript. Values represent the average of three independent assays.

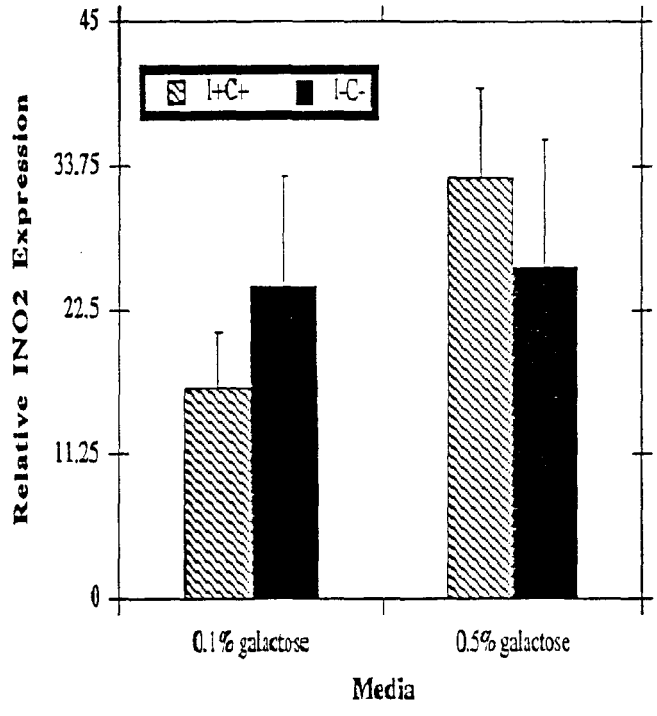
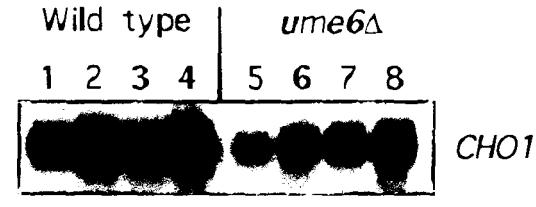
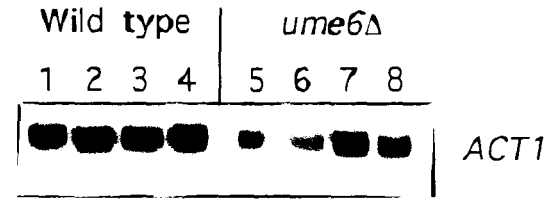

45
33.75
22.5
11.25
0
I+C+
I-C-
Relative INO2 Expression
0.1% galactose
0.5% galactose
Media

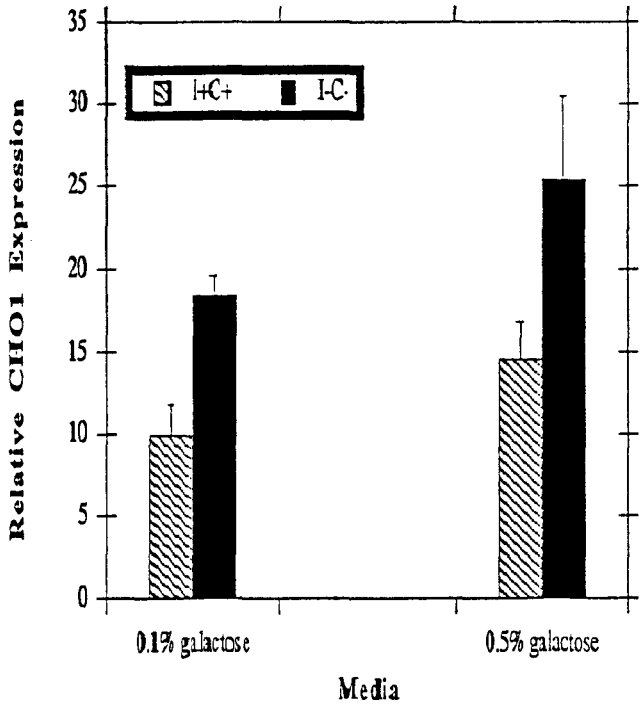
Figure 15. Northern blot hybridization demonstrating regulation of the *CHO1* gene in response to inositol and choline. Odd-numbered lanes represent repressing complete synthetic media containing 75μM inositol and 1mM choline (I+C+), while even-numbered lanes represent derepressing media (I-C-). Lanes 1,2: wild type strain BRS2011 (pGAL1-INO2) grown in 0.1% galactose. Lanes 3,4: Wild type strain BRS2011 (pGAL1-INO2) grown in 0.5% galactose. Lanes 5,6: *ume6*Δ strain BRS2013 (pGAL1-INO2) grown in 0.1% galactose. Lanes 7,8: *ume6*Δ strain BRS2013 (pGAL1-INO2) grown in 0.5% galactose. For comparison, the constitutively expressed *ACT1* transcript is shown.


Wild type
ume6Δ
1 2 3 4
5 6 7 8
CHO1


Wild type
ume6Δ
1 2 3 4
5 6 7 8
ACT1

(BRS2013) restores wild type regulation of *CHO1* by inositol and choline (Fig. 15) (Compare Fig. 9 and Fig. 16). Therefore, the defect in *CHO1* expression in the *ume6Δ* mutant strain is due to the underexpression of the *INO2* activator gene, and this defect can be overcome by increasing levels of the *INO2* transcript.

Figure 16. Increasing *INO2* transcript levels restores wild type regulation of *CHO1* in the *ume6*Δ strain (BRS2013). Expression of *CHO1* transcript from *ume6*Δ mutant strain (BRS2013) grown in complete synthetic media containing 0.1% or 0.5% galactose and either lacking (solid) or containing (hatched) 75μM inositol and 1mM choline. The amount of *CHO1*-specific cpm was determined through analysis of slot blot hybridizations and normalized for loading variations to the constitutively expressed *ACT1* transcript. Values represent the average of three independent assays.


Relative CHO1 Expression
35
30
25
20
15
10
5
0
I+C+
I-C-
0.1% galactose
0.5% galactose
Media

CHAPTER V
DISCUSSION

In *Saccharomyces cerevisiae*, regulation of the genes in the phospholipid biosynthetic pathway in response to the soluble lipid precursors inositol and choline has been shown to occur at the level of transcription of the *INO1* (Hirsch and Henry, 1986), *CHO1* (Bailis *et al.*, 1987), *CHO2* and *OPI3* structural genes (Gaynor *et al*, 1991; Kodaki *et al.*, 1991). Expression of these structural genes requires a common set of regulatory genes and a common *cis*-acting DNA element. The positive regulatory genes include *INO2* and *INO4*, which encode basic helix-loop-helix (bHLH) proteins (Hoshizaki *et al.*, 1990; Nikoloff *et al.*, 1992). The *INO2* and *INO4* gene products form a heterodimer that interacts with the UAS_{INO} element and is essential for *INO1* expression (Ambroziak and Henry, 1994; Donahue and Henry, 1981; Hoshizaki *et al.*, 1990; Nikoloff and Henry, 1994). In contrast, the products of the *OPI1* and *SIN3* regulatory genes act to repress the activities of the Ino2p and Ino4p proteins (Ashburner and Lopes, 1995b; Hudak *et al.*, 1994; Slekar and Henry, 1995). Strains bearing mutant alleles of these negative regulators display an inositol excretion phenotype (Opi^+ phenotype) which correlates with constitutive overexpression of the *INO1* gene (Greenberg et al., 1982; Hudak, 1994). In addition to the Opi^+ phenotype, these mutant strains constitutively overexpress the structural genes in the

phospholipid biosynthetic pathway, indicating that the Opi1p and Sin3p repressors function through the only common *cis*-acting element found in these promoters, the UAS_{INO} element (White *et al.*, 1991; Hudak *et al.*, 1994).

A computer-assisted DNA analysis of the *INO1* promoter region has identified a motif previously reported to play a role in repression of gene expression (Lopes *et al.*, 1993). This element was initially identified as an upstream repression sequence (URS1) in the promoter of the *CAR1* gene where it was required for the down regulation of *CAR1* gene expression during vegetative growth (Kovari *et al.*, 1990; Sumrada and Cooper, 1987). A functional URS1 element is also present in the promoters of several unrelated yeast genes including genes involved in meiosis (Bowdish and Mitchell, 1993; Buckingham *et al.*, 1990; Vershon *et al.*, 1992). Previous experiments also established that the *UME6* gene is absolutely required for URS1-mediated repression of genes involved in nitrogen catabolism (Park *et al.*, 1992) and early meiosis-specific genes during vegetative growth (Bowdish *et al.*, 1995; Steber and Esposito, 1995; Strich *et al.*, 1994). The *UME6* gene is also required for URS1-mediated meiotic induction of early, middle and late meiosis-specific genes (Bowdish *et al.*, 1995; Steber *et al.*, 1995).

The work described in this dissertation was aimed at understanding the mechanisms by which phospholipid biosynthesis is regulated by the product of the *UME6* gene. The results indicate direct and indirect regulation. In the direct mechanism, Ume6p acts to repress *INO1* transcription through the URS1 element in the *INO1* promoter. Indirect regulation occurs as Ume6p is required for full

expression of the *INO2* activator gene, which is required for expression of the phospholipid biosynthetic genes. This work is significant because it provides the first evidence of a regulatory role for *UME6* in both phospholipid biosynthesis and in the expression of the *INO2* regulatory gene.

Physical mapping of *UME6*

In this study, I determined the physical map location of the *UME6* gene to be the right arm of chromosome IV between the *pet14* and *hom2* genes. While I was determining the physical map location of *UME6*, the laboratory of Dr. Rochelle E. Esposito also mapped the location of the *UME6* gene using a combination of physical and genetic approaches. First, Esposito's laboratory mapped *UME6* to chromosome IV by hybridizing to a Southern blot of yeast chromosomes. Next *UME6* was further localized on chromosome IV through hybridization to filters containing subclones of chromosome IV. Finally, the chromosomal location of *UME6* was determined genetically through standard segregation analysis with markers known to be on the same chromosomal fragment. The genetic mapping experiments placed *UME6* 21 cM proximal to *pet14* and 23cM distal to *hom2* (Strich *et al.*, 1994), in good agreement to my results. The physical mapping of yeast genes is important for several reasons. In light of the yeast genome sequencing effort, the physical map locations of genes provide landmarks that can be used for the purposes of orientation and quality control. In addition, determining the physical map location of genes may identify discrepancies between the physical map location and the genetic map

location of a gene. Genetic mapping of yeast genes is based on recombination frequencies, and the identification of differences in the physical and genetic map locations of a gene may be useful in identifying interesting recombination events. Finally, the physical mapping of yeast genes may provide information on the overall organization of genes on chromosomes, perhaps revealing clues to the differential expression of genes.

UME6 is required for proper regulation of phospholipid biosynthetic genes

Consistent with the Opi+ phenotype (Fig. 7), I found that the *INO1* gene was overexpressed in a *ume6*Δ mutant strain, grown under repressing conditions (presence of inositol and choline), to levels seen in the wild-type strain under derepressing conditions (Fig. 8). However, despite the elevated expression of *INO1* in the *ume6*Δ mutant, *INO1* expression was still modestly responsive to the presence of inositol and choline (Fig. 8), most likely due to the action of the Opi1p repressor at the *INO1* promoter.

Previous experiments have demonstrated that a mutation in the negative regulator *OPI1* leads to constitutive overexpression of the *CHO1* gene (White *et al.*, 1991). I demonstrated in this study that the other co-regulated structural genes in the phospholipid biosynthetic pathway (*i.e.*, the *CHO2*, and *OPI3* genes) are similarly affected by the *opi1*Δ mutant allele (Fig. 9). Similarly, a mutation in the negative regulator *SIN3* leads to constitutive expression of the co-regulated genes in the phospholipid biosynthetic pathway at derepressed levels; however, they are not overexpressed as in the

case of an *opi1* mutant strain (Hudak *et al.*, 1994). By contrast, a *ume6*Δ mutation renders the *CHO1*, *CHO2*, and *OPI3* genes constitutive, but at levels identical to those observed for a wild-type strain under repressed conditions (Fig. 9). Thus, my results identified a novel positive regulatory role for the *UME6* gene on expression of the *CHO1*, *CHO2*, and *OPI3* genes. Therefore, the *OPI1* and *SIN3* genes act to reduce expression of the phospholipid biosynthetic genes, but the *UME6* gene functions to reduce *INO1* expression and is required for induction of *CHO1*, *CHO2*, and *OPI3* gene expression.

A *ume6*Δ mutation causes physiological changes in yeast cells

a). A *ume6*Δ mutant strain has an Opi$^+$ phenotype

Regulation of phospholipid biosynthesis is a tightly controlled process. Proper regulation requires the products of many different regulatory proteins and promoter elements. The *de novo* production of inositol by I1PS (product of the *INO1* gene) is under the tightest regulatory control of the phospholipid biosynthetic genes (Lopes and Greenberg, in press). Initially, in a screen for inositol-excreting mutants (Opi$^+$ phenotype), four genetic loci were isolated (Greenberg *et al.*, 1982). Two of the mutations were subsequently identified, and one of the mutations identified the *OPI1* negative regulatory gene (Greenberg *et al.*, 1982). Unexpectedly, this genetic screen also identified the *OPI3* structural gene (Greenberg *et al.*, 1982), which encodes a methyltransferase in the PC biosynthetic pathway (McGraw and Henry, 1989). Studies of the regulation of phospholipid

biosynthesis in *cho1* (Letts and Dawes, 1983), *cho2* (Hirsch and Henry, 1986), and *opi3* (McGraw and Henry, 1983) mutant strains have revealed that ongoing synthesis of PDME or PC is required for transcriptional regulation in response to inositol. Mutations in either *CHO1*, *CHO2* or *OPI3* will result in the Opi$^+$ phenotype; however, this phenotype is conditional, unlike an *opi1* mutation. In each type of mutant (*cho1*, *cho2*, or *opi3*), repression of *INO1* transcription in response to inositol is restored if PC biosynthesis is reestablished by adding precursors that enter the PC pathway downstream of the respective metabolic lesion (Nikoloff and Henry, 1991). The Opi$^+$ phenotype also identified a mutant allele of the *CDG1* gene, whose product produces CDP-DG, the direct source of the phosphatidyl moiety in the synthesis of PI and PS (Henry, 1982; Steiner and Lester, 1972). As an attempt to compensate for the reduced levels of CDP-DG inside the cell, the *cdg1* mutant strain overproduced both IPS and PSS, perhaps in an attempt to drive phospholipid biosynthesis (Klig *et al.*, 1988). In addition, a null mutation in the regulatory gene *SIN3* (Hudak, 1994) also results in the Opi$^+$ phenotype, corresponding to constitutive expression of the *INO1* gene.

Results from this study demonstrate that a strain harboring a *ume6*Δ mutation shares the Opi$^+$ phenotype since it excretes inositol into the growth media (Fig. 7). The Opi$^+$ phenotype of a *ume6* mutant has proven to be very useful to the yeast community studying *UME6* function. Ideally, a Southern blot is used to verify a deletion or disruption of a target gene. Unfortunately, the difficulty in deleting or disrupting *UME6* from the yeast genome (J. Jackson and C. Steber, unpublished observations) has made this approach

inadequate, since a large number of possible mutants do not contain a mutant *ume6* allele. As an alternative, a disruption or deletion mutation in *UME6* can be verified by transforming a reporter plasmid into the strain in question and looking for misregulation of the reporter. While successful, this strategy proves to be time-consuming. Through the use of the Opi$^+$ plate assay described in this study, mutations in the *ume6* gene can be followed in a less time-consuming manner because the need for strain construction is eliminated.

b). A *ume6*Δ mutant strain has a novel phospholipid composition

Mutations in the positive regulatory genes *INO2* and *INO4* (Lowey and Henry, 1984) and mutations in the negative regulatory genes *OPI1* (White *et al.*, 1991) and *SIN3* (Hudak *et al.*, 1994) have contrasting pleiotropic effects on the expression of the phospholipid biosynthetic genes. Mutations in the positive regulatory genes have two consequences: inositol auxotrophy due to a lack of *INO1* expression (Donahue and Henry, 1981); and defects in the synthesis of PC due to a failure in derepression of the PC biosynthetic genes (White *et al.*, 1991b). A result of misregulation of the biosynthetic genes is a change in the phospholipid composition of the cell membranes. In a wild type strain, in the presence of inositol and choline, the relative level of PI in membranes increases from approximately 17% to 28%, and this change occurs in conjunction with small decreases in the levels of PS and PE and a drop in PC from 39% to 32% (Loewy and Henry, 1985). As discussed above (Figure 1),

S. cerevisiae has a salvage pathway it can utilize to produce phospholipids, and this pathway is responsible for the changes in phospholipid composition when cells are grown in the presence of inositol and choline. In the absence of inositol and choline, the inositol used to make PI is synthesized inside the cell by the IPS enzyme (product of the *INO1* gene). When inositol is present, transcription of the *INO1* gene is repressed, IPS levels decrease, and the inositol used to make PI must come from outside the cell. The increase in PI levels in the presence of inositol is due to the combined action of the inositol transporters (*ITR1* and *ITR2*) and the PIS enzyme, product of the *PIS1* gene, whose transcription is unresponsive to inositol and choline. Since the PIS enzyme and the PSS enzyme compete for CDP-DG, an increase PIS activity will lead to a decrease in PSS activity, resulting in decreased production of PC (Lopes and Greenberg, in press).

In *ino2* or *ino4* mutant strains, phospholipid composition can only be determined in media containing inositol since they are inositol auxotrophs (Donahue and Henry, 1981). In comparison to a wild type strain, an *ino2* or *ino4* mutant strain exhibits an increase in PI (37% to wild type 28%) and more dramatic increases in the intermediates in the PC biosynthetic pathway (PE: 25% compared to wild type 12%; PDME: 9% to 2%); however, ultimately the *ino2* and *ino4* mutant strains produce approximately 3-fold less PC than a wild type strain (13% compared to wild type 40%) (Loewy and Henry, 1985). The defect in phospholipid composition in an *ino2* or *ino4* mutant strain is due to the requirement of *ino2* and *ino4* for

expression of the phospholipid biosynthetic genes in both the PI and PC pathways.

In contrast to *ino2* and *ino4* mutant strains, the phospholipid composition of an *opi1* mutant strain resembles that of a wild type strain grown under repressing conditions (see above) (Klig *et al.*, 1985). In an *opi1* mutant strain, in the presence or absence of inositol and choline, the relative levels of phospholipids are as follows: PI, approximately 27%; PS, 7%; PE, 25%; and PC, 34% (Klig *et al.*, 1985). In an *opi1* mutant strain, regardless of media composition, the overexpression of *INO1* results in excess amounts of inositol being synthesized, which saturates the PIS enzyme, resulting in high levels of PI. The overexpression of the inositol transporter *ITR1* in an *opi1* mutant most likely has no effect on PI levels because the PIS enzyme is already saturated. Therefore, the primary cause for the increase in PI levels in an *opi1* mutant strain is the overexpression of the *INO1* gene.

Despite the pleiotropic effect of a *sin3* point mutation on expression of the phospholipid biosynthetic genes, this resulted in no change in phospholipid composition (J. Lopes, unpublished results). The lack of measurable difference in phospholipid composition in a *sin3* mutant strain is most likely due to leakiness of the point mutation, since the point mutation also lacked the Opi$^+$ phenotype demonstrated by a *sin3*Δ mutation (Hudak, 1994).

In this study I determined the phospholipid composition of a *ume6*Δ mutant strain (Table 2). In comparison to other regulatory mutants, a *ume6*Δ mutant strain has a unique phospholipid composition. In a wild type strain, I found the amount of PI to

increase from approximately 15% to 24% when inositol is added to the growth media, while in a *ume6*Δ strain, the amount of PI was at 19% regardless of media composition (Table 2). This intermediate level of PI can be explained through two observations. One pathway for PI biosynthesis utilizes exogenous inositol which is transported into the cell primarily by the product of the *ITR1* gene. Transcription of the *ITR1* gene is regulated by inositol and choline and requires the *INO2* and *INO4* genes (Lai and McGraw, 1994). Since *INO2* expression is decreased in a *ume6*Δ mutant strain (Fig. 10), expression of *ITR1* may also be reduced, limiting the import of inositol into the cell. Compensating for the decrease in inositol transport, a *ume6*Δ mutant strain overexpresses the *INO1* gene (Fig. 7), whose product ultimately leads to the production of inositol. The difference in PI levels in a *ume6*Δ mutant strain, as compared to PI levels in an *opi1* mutant strain, is most likely due to the degree of overexpression of the *INO1* gene. An *opi1*Δ mutant strain produces more *INO1* than the *ume6*Δ mutant strain (Fig. 8). Consequently, the *opi1*Δ mutant strain produces excess endogenous inositol and additional transport of inositol into the cell has no effect because the PIS enzyme is saturated. In contrast, a *ume6*Δ mutant strain produces less endogenous inositol than an *opi1*Δ mutant strain, and the PIS enzyme is not saturated. Therefore, inositol transport could raise the PI levels in a *ume6*Δ mutant strain, but expression of the inositol transporter, *ITR1*, is probably reduced because of the low levels of *INO2* expression (Fig. 10).

In addition to an intermediate level of PI, a *ume6*Δ mutant strain also has an intermediate level of PC compared to a wild type

strain. I determined the levels of PC in a wild type strain to change from approximately 40% to 34% when inositol and choline are added to the growth media (Table 2). In contrast, a *ume6*Δ strain had constant levels of PC, approximately 37%, regardless of media composition (Table 2). As is the case with PI, an explanation of this intermediate level of PC probably lies within the salvage pathway of phospholipid biosynthesis. In the absence of inositol and choline, the increased activity of the PIS enzyme making PI (due to overproduction of inositol), uses up more of the available CDP-DG which is also needed for the *de novo* PC biosynthetic pathway (Lopes and Greenberg, in press). Reduced availability of CDP-DG and expression of the PC biosynthetic genes at wild type repressed levels (Fig. 9) ultimately leads to reduced PC levels. In the presence of inositol and choline, the levels of PE and PC synthesized by the salvage pathway are presumably reduced due to possible underexpression of the choline transporter (*CTR1*), which is dependent on *INO2* for expression (Li and Brendel, 1993).

UME6 is required for full expression of the *INO2* positive regulatory gene

The positive regulatory role for the *UME6* gene on *CHO1*, *CHO2*, and *OPI3* expression suggested that *UME6* may be required for proper expression of the *INO2* and *INO4* transcriptional activator genes. It is known that expression of an *INO2-cat* fusion gene is regulated in response to inositol and choline, while expression of an *INO4-cat* fusion construct is known to be constitutive (Ashburner and Lopes, 1995). Moreover, in an *opi1*Δ mutant, the *INO2-cat* fusion

gene is constitutively overexpressed whereas expression of the *INO4-cat* gene is unaffected (Ashburner and Lopes, 1995). In contrast to the *opi1*Δ effect, I found *INO2-cat* expression in a *ume6*Δ mutant strain was markedly decreased under both repressing and derepressing conditions when compared to a wild-type strain (Fig. 10). Thus, the *UME6* gene had a positive regulatory role in transcription from the *INO2* promoter. This decreased expression of the *INO2* activator gene in the *ume6*Δ strain correlates with the effect of the *ume6*Δ mutation on expression of the *CHO1*, *CHO2*, and *OPI3* genes.

The *INO2* promoter contains a region required for regulation by *UME6*

Earlier in this study, data was presented demonstrating a decrease in expression of a reporter gene (*cat*) driven by the *INO2* promoter in a *ume6*Δ strain (Fig. 10). A computer-assisted search of the *INO2* promoter region did not reveal the presence of the URS1 element. Therefore, an analysis of the *INO2* promoter region was conducted to identify *cis*-acting elements responsible for the *UME6*-dependent regulation of *INO2* gene expression. Results of an *INO2* promoter deletion analysis revealed that a 150 bp region of the *INO2* promoter, which includes a potential UAS_{INO} element (at -134 relative to the AUG initiation codon) and the putative transcriptional start site (at -106), was important for regulation of *IN02-cat* expression by *UME6* (summarized in Fig. 13). Curiously, within the region required for *UME6* function is a second potential AUG translational start codon found 17bp upstream from the Ino2p

translational start codon. If translation occurred from this upstream AUG, the result would be a potential open reading frame (ORF) of 57 nt (19 amino acids) which would overlap the Ino2p ORF. Currently it is not known whether or not this upstream ORF is translated. In addition, this leader region is predicted to form a stem-loop structure which could also affect *INO2* regulation (J. Lopes, unpublished observations). Translational control in yeast through the use of upstream ORF's has previously been demonstrated for the *GCN4* (Hinnebusch, 1984) activator gene and the *CPA1* gene which encodes a subunit of an arginine pathway enzyme (Werner, *et al.*, 1987). Coincidentally, the leader of the *UME6* mRNA also contains 5 short upstream ORFs which may play a role in regulating *UME6* levels within the cell (Strich *et al.*, 1994).

A large 3' deletion in the *INO2* promoter from (-1 to -150), which removes the region important for *UME6*-mediated regulation, resulted in constitutive low level expression of the *INO2-cat* gene in both a wild type and *ume6*Δ mutant strain (Fig. 13). However, since the resulting CAT activity from this construct is essentially at background levels, no conclusions can be drawn concerning regulation by *UME6*.

A deletion which removed the first 50 bp of the promoter (-1 to -50, including the upstream AUG) resulted in a constitutively high level of expression from the *INO2*-cat gene in a wild type and *ume6*Δ strain, as was the case with another construct which removed the 50 bp around the UAS_{INO} element (Fig. 13). The similarity in CAT activity between a wild type strain and *ume6*Δ mutant strain indicates that these regions may be important for *UME6* regulation of

INO2 gene expression. However, the relative importance of these promoter segments in the regulation of *INO2* gene expression by *UME6* is unclear. Since both promoter deletions result in similar levels of *INO2-cat* expression, one possibility is that *UME6*-dependent regulation requires the presence of both promoter elements. Another possibility is that only one of the promoter segments is required for *UME6*-dependent regulation, but deletion of the other segment is epistatic, and thus will mask the regulation by *UME6*. Clearly, a more detailed analysis of the *INO2* promoter will be required to eliminate one of these possibilities.

Induction of *CHO1* is *IME1*-independent

Recently, experiments have demonstrated a direct role for *UME6* in the meiotic induction of early meiosis-specific genes (Bowdish *et al.*, 1995; Steber and Esposito, 1995). The ability of *UME6* to activate transcription has also been shown to be dependent on the product of the *IME1* gene, a known inducer of meiosis (Bowdish *et al.*, 1995). Based on these experiments, I initially reasoned that if *UME6* is required for induction of *INO2* gene expression, the activation might be *IME1*-dependent. Consequently, if *IME1* was required to convert Ume6p to an activator needed for full *INO2* expression, an *ime1*Δ mutant strain should have the same defect in target gene expression as a *ume6*Δ mutant strain. In this study, I demonstrated that the presence or absence of *IME1* has no effect on *CHO1* gene expression (Fig. 11). Therefore I conclude that *IME1* plays no role in the regulation of phospholipid biosynthesis.

UME6 represses *INO1* transcription through the URS1^{INO1} element

Many yeast genes in unrelated systems are known to contain a URS1 element in their promoters, and to require this element for repression of gene expression. In this report, I directly examined the role of the URS1^{INO1} element in repression of *INO1* gene expression. We created two fusions of the *INO1* promoter to the *cat* reporter gene, which were identical except for a mutation of the URS1^{INO1} element of one reporter construct. The reporter constructs containing the mutation in the URS1^{INO1} element yielded constitutive CAT activity (Fig. 12) regardless of strain genotype, indicating that the URS1^{INO1} element is crucial for repression of *INO1*. Curiously, the wild-type strain yielded levels of CAT activity that were higher than in the *ume6*Δ strain, but this can be explained by the lower amount of *INO2* activator present in the *ume6*Δ strain (Fig. 10). Since there was no synergy between the *ume6*Δ mutation and the mutant URS1 element, I concluded that *UME6* regulates *INO1* gene expression primarily through the URS1^{INO1} element. Regulation involving the URS1 element is quite complex and can involve several different system-specific players. In the case of the *CAR1* gene, which is involved in nitrogen metabolism, the *UME6* gene is absolutely required for URS1-mediated repression (Park *et al.*, 1992), although it is the products of the *BUF1* and *BUF2* (*RPA1* and *RPA2*) genes that actually bind to the URS1^{CAR1} element (Luche *et al.*, 1993). However, in the case of the meiosis-specific gene *SPO13*, experiments using an MBP-Ume6p fusion protein have demonstrated direct binding of the Ume6p fusion protein to the URS1^{SPO13} element (Strich

et al., 1994). In addition, full repression of the *SPO13* gene also requires the product of the *SIN3* gene (Vidal *et al.*, 1991), which is not required for repression of *CAR1* (Park *et al.*, 1992). Since repression of *INO1* also requires the product of the *SIN3* gene, the repression system for *INO1* is best compared to the repression of *SPO13*.

Elevated expression of *INO2* from the *GAL1* promoter restores transcriptional regulation of *CHO1* expression in a *ume6*Δ strain

Data described above showed that expression of a reporter gene (*cat*) driven by the *INO2* promoter was decreased in a *ume6*Δ mutant strain (Fig. 10). The effect of a *ume6*Δ mutation on *INO2-cat* gene expression was consistent with the effect of a *ume6*Δ mutation on expression of the *INO2* target genes *CHO1*, *CHO2*, and *OPI3* (Fig. 9). This raised the possibility that either Ume6p acted directly at the promoters of these target genes, or that the defect in expression of these genes in a *ume6*Δ mutant strain was due to a decrease in the expression of the *INO2* activator gene. Since the promoters of these *INO2* target genes do not contain an identifiable URS1 element, I chose to examine the contribution of *INO2* toward their regulation. To determine the role of *INO2* expression in the regulation of these genes in a *ume6*Δ mutant strain, I used a previously characterized system (Ashburner and Lopes, 1995b) that uncoupled *INO2* expression from the inositol/choline response by placing it under the control of the galactose-inducible *GAL1* promoter.

Previous experiments have demonstrated that transcription of *INO2* in a wild type strain harboring the *GAL1-INO2* fusion, is unresponsive to inositol and choline, and increases with increasing levels of galactose in the growth media (Ashburner and Lopes, 1995b). In addition, expression of *INO2* from the strong *GAL1* promoter results in higher levels of *INO2* expression compared to a wild type strain. However, even though *INO2* transcription increased, expression of the *INO2* target genes *INO1* and *CHO1* was still regulated by inositol and choline in a manner dependent on the product of the *OPI1* gene (Ashburner and Lopes, 1995b). I used this system to determine whether or not increasing the levels of *INO2* mRNA in a *ume6*Δ mutant strain would restore regulation of the *CHO1* gene. I chose to use concentrations of 0.1% and 0.5% galactose in the growth media because previous experiments have demonstrated a linear relationship between the amount of *INO2* and the amount of target gene expression at these concentrations (Ashburner and Lopes, 1995b). I first demonstrated that a *ume6*Δ mutation did not dramatically affect transcription of the *GAL1-INO2* fusion gene. The data demonstrate that *GAL1-INO2* expression is unresponsive to inositol and choline in a *ume6*Δ mutant strain (Fig. 14) as it was in a wild type strain (Ashburner and Lopes, 1995b). I next assayed *CHO1* gene expression in a *ume6*Δ mutant strain containing the *GAL1-INO2* fusion gene. The data demonstrate that increasing the amount of *INO2* expression in a *ume6*Δ mutant strain eliminates the defect in *CHO1* gene expression (Fig. 9, 15, and 16). As demonstrated previously in a wild type strain (Asburner and Lopes, 1995b), increasing *INO2* gene expression also increases *CHO1* gene

expression (Fig. 14,15, and 16). Corresponding with the increased *CHO1* gene expression, is the return of *CHO1* gene regulation by inositol and choline, indicating that the inositol regulatory machinery is still intact in a *ume6*Δ mutant strain. Therefore, the defect in expression of the target genes *CHO1*, *CHO2*, and *OPI3* in a *ume6*Δ mutant strain is a result of decreased expression of *INO2*.

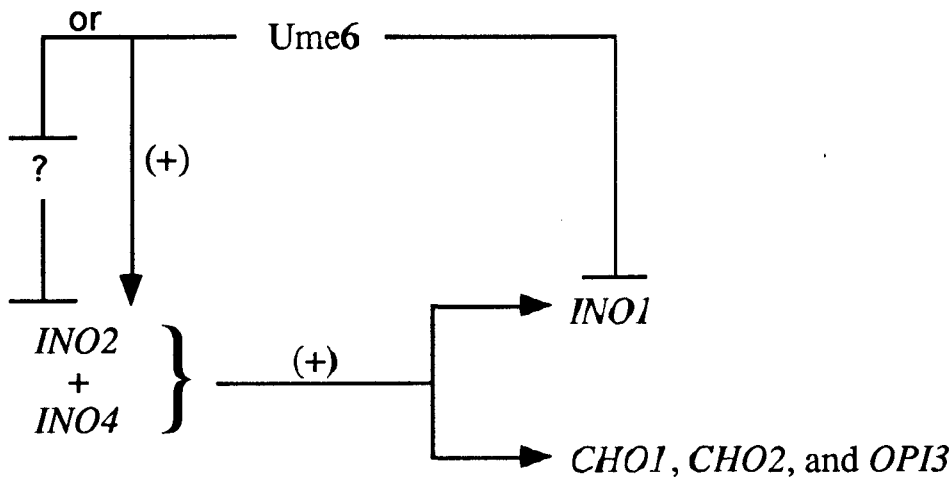
This above result seems to be in conflict with the effect of a *ume6*Δ mutation on *INO1* expression. In a *ume6*Δ mutant strain, *INO1* is expressed at levels equal to or greater than the wild type derepressed levels (Fig. 8), while the *CHO1*, *CHO2*, and *OPI3* genes are expressed constitutively at wild type repressed levels (Fig. 9). One explanation for this difference concerns the promoters of these genes. Of this group, only the *INO1* promoter contains two functional UAS_{INO} elements (Lopes *et al.*, 1991) and a functional URS1 element (Lopes *et al.*, 1993). The repressive capability of the $URS1^{INO1}$ element must be able to overcome the activation of two functional UAS_{INO} elements. Therefore, when *INO2* levels are lowered due to a *ume6*Δ mutation, the *INO1* promoter has the greatest activation potential because of the two functional UAS_{INO} elements and the inactivation of $URS1^{INO1}$ function which requires the product of the *UME6* gene.

Model for *UME6* regulation of phospholipid biosynthesis

I propose the following model to explain the role of the *UME6* gene product on expression of the phospholipid biosynthetic genes (Fig. 17). The model predicts that the Ume6p protein functions to directly inhibit transcription of the *INO1* gene through the $URS1^{INO1}$

element and that it may stimulate transcription of the *INO2* gene either directly or indirectly. The direct mechanism would require that Ume6p function as a transcriptional activator of the *INO2* gene. This mechanism is difficult to envision since the *INO2* promoter lacks any URS1-like sequences and since it has been reported that Ume6p was not capable of activating transcription in a diploid cell during vegetative growth or in the absence of Ime1p (Bowdish *et al.*, 1995). Therefore, this mechanism would require that Ume6p function as a URS1-independent, *IME1*-independent, haploid-specific transcriptional activator. I currently favor the indirect mechanism which predicts that Ume6p would function to repress a negative regulator of *INO2* transcription. This indirect mechanism would not require the presence of a URS1 element in the *INO2* promoter and would not be dependent on the *IME1* gene nor diploid-dependent.

Figure 17. Model for regulation of phospholipid biosynthesis by *UME6*.


or
Ume6
?
(+)
INO1
INO2
+
INO4
(+)
CHO1, CHO2, and OPI3

Future directions

There are several areas to study in order to further understand the role of *UME6* in the regulation of phospholipid biosynthetic gene expression. Among these is to determine the *cis*-acting element required for *UME6* regulation of *INO2* expression. The deletion analysis of the *INO2* promoter revealed a region important for this regulation, but this region contains several possible important elements. Creating point mutations in these elements could narrow down the region important for *UME6* regulation of *INO2* gene expression.

A further test of the model also needs to be done to determine if *UME6* acts directly or indirectly to regulate *INO2* gene expression. The data indicates that the *UME6* gene is required for induction of *INO2* gene expression. If Ume6p acts directly at the *INO2* promoter to activate *INO2* expression, a previously isolated *ume6* mutant (*rim16-12*) which fails to activate meiotic gene expression might also fail to activate *INO2* gene expression. If Ume6p acts indirectly by repressing transcription of a repressor of *INO2* gene expression, this mutant would still be able to repress transcription of the unidentified repressor and *INO2* expression would be unaffected.

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